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The Effect of Isocitrate Dehydrogenase on the Epigenetics of Human Mitochondrial DNA

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A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science at Virginia Commonwealth University.

by

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Abstract

THE EFFECTS OF ISOCITRATE DEHYDROGENASE ON HUMAN MITOCHONDRIAL EPIGENETICS

By John Andrew Strang, B.S.

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Aberrant metabolism has become an increasingly interesting area of cancer biology. In many cancers including lower grade glioma, glioblastomas and some leukemias, a mutation in the metabolic enzyme Isocitrate Dehydrogenase (IDH), has been found in more than 70% of cases and has been shown to lead to a distinct hypermethylator phenotype. IDH commonly converts isocitrate to alpha-ketoglutarate in normal cell metabolism. Three isoforms of this enzyme are found in humans: IDH1, IDH2 and IDH3. Studies on IDH1, the cytosolic isoform, have revealed that mutations in the enzyme's binding site lead to a novel gain of function: the synthesis of an oncogenic metabolite, 2-hydroxyglutarate (2HG). 2HG competitively inhibits alpha-ketoglutarate dependent enzymes such as the TET 5-methylcytosine (5mC) oxygenases and histone demethylases. These oxygenases are responsible for the hydroxymethylation (5hmC) of cytosine residues, ultimately leading to demethylation and gene re-expression. Thus, mutant IDH may lead to an elevation in 5mC levels producing the hypermethylator phenotype described.

A similar gain-of-function mutation in IDH2, the mitochondrial isoform of IDH1, has been associated with leukemias and gliomas lacking IDH1 mutations. Mutations in IDH1, IDH2 and TET2 are mutually exclusive, and each yields a similar hypermethylator phenotype. IDH2,

along with IDH3, is primarily involved in the TCA cycle and energy production for the cell. Recently, the Taylor lab has uncovered evidence of 5mC and 5hmC residues in mitochondrial DNA, established and maintained by mtDNMT1 and TET2. Changing levels of mtDNMT1 appears to alter the patterns and levels of mtDNA transcription from the mitochondrial genome. We hypothesized that mutant IDH would produce a similar effect on the mitochondrial genome as that found in the nuclear genome and result in a decrease in the level of 5-hydroxymethylcytosine, as well as a subsequent increase in the level of 5-methylcytosine caused by the competitive inhibition of the TET enzymes by 2-hydroxyglutarate accumulation. Using molecular biology techniques such as Western blots and MeDIP (methylated DNA immunoprecipitation) I aim to uncover the role of IDH mutation on mitochondrial DNA methylation and its effect on energy production in mammalian cells.

Chapter 1: Introduction

The Study of Epigenetics in Cancer

Epigenetics is defined as the study of changes in gene expression independent of changes in the DNA sequence itself (34). These genetic changes can be passed down from generation to generation within a species and work to silence genes in a controlled and regulated way. Mammalian species have evolved multiple regulatory pathways involving epigenetics including the silencing of large portions of one of the X chromosomes in females during X linked inactivation, differentiation of stem cells during development (28), and genomic imprinting. Epigenetics can be subdivided into three major categories: Nucleosome remodeling, Histone modifications and DNA methylation (88).

Of the three categories, DNA methylation is the most thoroughly studied. DNA methylation involves the transfer of a methyl group from S-adenosylmethionine (SAM) to the fifth carbon position of a cytosine residue in a cytosine guanosine (CpG) dinucleotide. There are many CpG rich areas found in the genome called "CpG islands" which are defined as areas of about 200 base pairs, containing an observed-to-expected CpG ratio of greater than 60%. These CpG rich areas comprise about 40% of the promoters in mammalian genomes (35) with more than 70% of human gene promoters carrying a large CpG content. Promoter CpG methylation in mammals ultimately leads to the repression of transcription from that promoter (39). The enzymes responsible for cytosine methylation in the human genome make up the family of DNA methyltransferases (DNMTs). There are three catalytically active DNMT isoforms in the mammalian genome that work to ensure that proper cytosine methylation occurs: DNMT1, DNMT3a and DNMT3b. DNMT1 is known as a maintenance methyltransferase and works to

maintain the methylation patterns of the genome during DNA replication. Both DNMT3a and DNMT3b work as de novo methyltransferases that place new methyl groups on cytosine residues when necessary such as in early development (58, 88). A catalytically inactive DNA methyltransferase, DNMT3L, also exists that establishes maternal genomic imprinting during gametogenesis (9). Another DNA methyltransferase family member, originally labeled as DNMT2, has been shown to mutilate the transfer RNA molecule that encodes for aspartic acid and is therefore functionally distinct from the other DNMTs in that it can methylate RNA (23).

Over centuries, DNA methylation of the mammalian genome has lead to substantial CpG dinucleotide depletion (27). Once methylated, cytosine residues can be deaminated spontaneously or by activation-induced cytosine deaminase (AID) to thymine. Persistence of thymine in place of cytosine results in CpG to TpG mutations, and a decrease in the total amount of cytosine residues able to be methylated in the context of CpG dinucleotides. However, small areas of the genome, typically found in the promoter areas of many genes have retained close to the expected amount of CpG dinucleotides, largely because they are resistant to cytosine methylation and these areas have been designated CpG islands. In normal differentiated cells, the epigenome is generally described as unmethylated at these CpG island sites with concomitant methylation of non-island CpG dinucleotides found usually within the body of the gene (Figure 1-1). These non-island CpG sites can also be found in repetitive areas within the genome as well as around inserted viral sequences and transposons which can move to different areas of the genome randomly. This pattern allows for ample gene transcription from the promoter start site and represses unwanted transcription at these irregular elements of the cellular genome which could do harm to the cell. In contrast to this pattern of normal gene transcription, in cancer the epigenetics of the cell are altered to form an entirely different pattern of CpG methylation. A

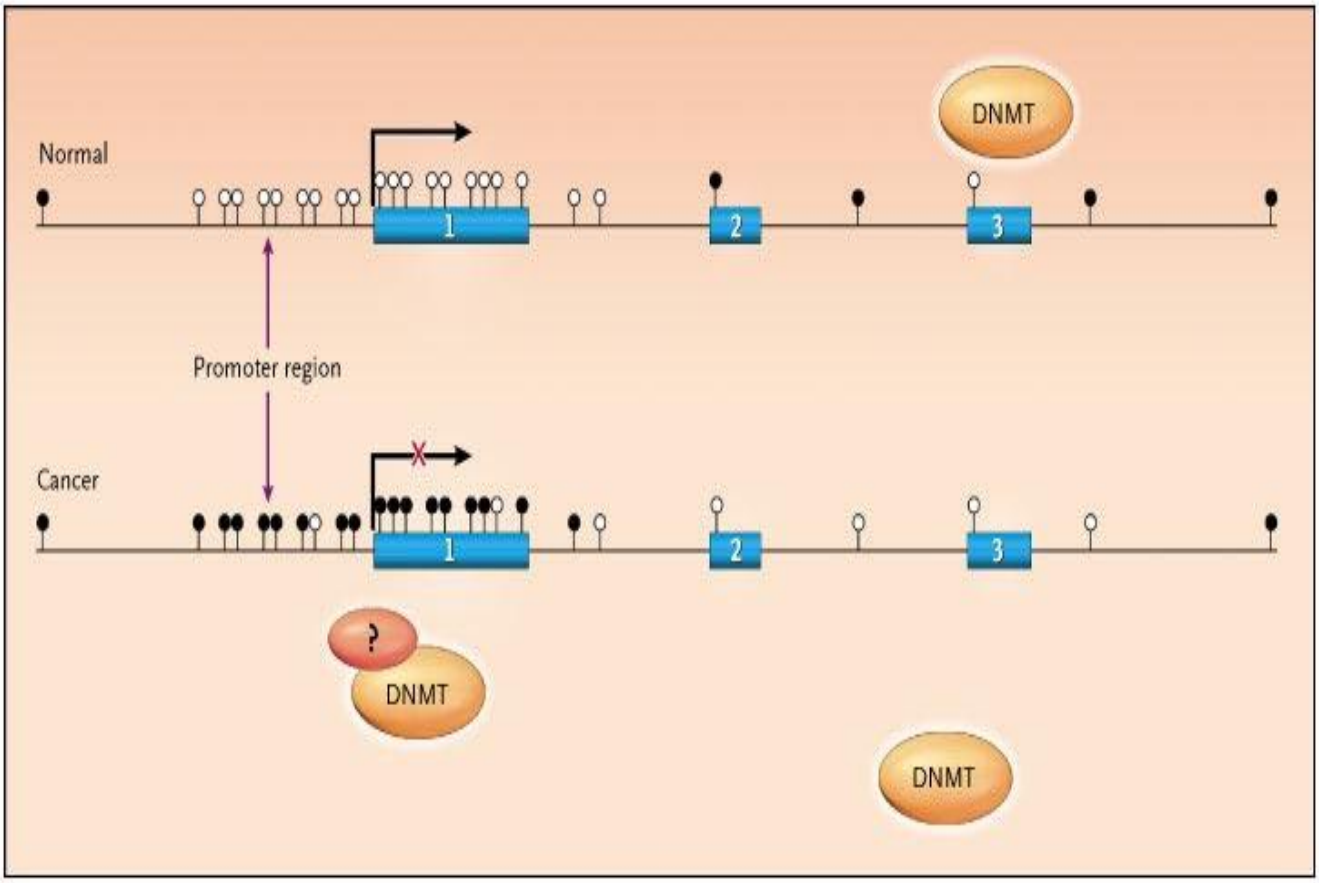

Figure 1-1: Diagram representing differential patterns of methylation in normal and cancerous cells. In normal cells, genes are characterized by hypomethylation at CpG island promoter regions, which account for about 40% of gene promoter areas, and CpG methylation within the gene body. Conversely, in cancer cells, the epigenomic pattern is marked by promoter CpG island hypermethylation and a subsequent hypomethylation of CpG dinucleotides within the gene body. Figure adapted from Herman, 2003, New England Journal of Medicine

distinctive pattern is produced wherein the genome becomes globally hypomethylated with subsequent hypermethylation of the CpG islands found at gene promoters (63). This pattern generally leads to a large increase in gene transcription from normally repressed genes (oncogenes) and an ensuing repression of gene transcription levels from normally active genes (tumor suppressors). The genomic hypomethylation phenomenon can lead to greater genomic instability and chromosomal rearrangement which in turn can lead to the uncontrolled activation of growth promoting genes, such as RAS, and the loss of imprinting in many cells. This aberrant growth has come to be known as one of the hallmarks of cancer (25). The abnormal methylation pattern has become a field of intense study as epigenetic inactivation has succeeded mutational inactivation as the most common insult found in the cancer genome. This hypermethylation is so integral to the development of cancer that a distinctive phenotype, the CpG Island Hypermethylator phenotype (CIMP), discovered in the 1990s, was confirmed by showing that a subset of CpG islands is coordinately methylated in tumors (74). The subset of genes most commonly affected in the CIMP phenotype are those that are targets for polycomb group proteins which are essential for epigenetic silencing. Typically genes involved in cell cycle progression, apoptosis, DNA repair and growth factor response, such as Rb, caspase 8, BRCA1 and ER respectively, are methylated so that the cell does not respond to arrest signals and continues to grow leading to tumorigenesis (5 and 39). The rise in oncogenes that promote growth and the fall in tumor suppressing genes that inhibit expansion both work to define cancer in many tumor types.

Many studies have pointed to the DNA methyltransferase, DNMT1, the maintenance methyltransferase, as the culprit of abnormal methylation patterns during tumorigenesis (27). This enzyme methylates newly synthesized hemimethylated daughter strands after replication to

ensure stably inherited methylation of the genome from generation to generation. However, this enzyme interacts with the other DNMTs, the *de novo* methyltransferases DNMT3A and DNMT3B, to establish patterns of methylation and all three are required for normal embryonic development, making a single cause of aberrant methylation in cancer difficult to pinpoint. Other studies have shown that DNMTs work with histones and the chromatin surrounding CpG island promoters to activate and repress gene transcription. In normal cells, where promoters are unmethylated at CpG sites, nucleosomes are widely spaced. This spacing is achieved by histone tail modifications, such as acetylation of key lysine residues on histone H3, which open up the nucleosomes allowing access by transcription factors to the promoter for gene transcription activation. Contrasting this, in cancer where CpG island promoter regions are almost exclusively methylated, the nucleosomes are condensed prohibiting transcription factors from binding to the promoter region of this gene. These regions are also typically associated with histone deacetylases and histone methylation marks at lysine 9 of histone H3 which are typically indicative of gene repression and inactivation (27). The study of epigenetics is critical to understanding tumorigenesis and many connections between histone modifications, nucleosome remodeling and cytosine methylation have been made in regards to gene transcription regulation. It is now impossible to think of cancer without understanding epigenetic processes as well. In this thesis, we have focused on cytosine modifications and the relationship they play in transcription to alter gene expression as it relates to reprogramming metabolism.

The Role of Isocitrate Dehydrogenase in the Tricarboxylic Acid Cycle

Multiple enzymes of the TCA cycle have been shown to be implicated in the progression of cancer including Succinate Dehydrogenase (SDH) (67) and Fumarate Hydratase (FH) (31). These enzymes, in normal tissues, typically contribute to ATP production

via the TCA cycle and once mutated, such as in cancer, lead to the accumulation of metabolic intermediates within the cell and aberrant cellular metabolism. This build up leads to an increase in the production of hypoxia inducible factor α (HIFα) which works to facilitate the cell's response to a hypoxic environment. This response contributes to the processes of angiogenesis, apoptosis and metabolism so that the cell can survive in a low-oxygen situation. With HIF1α constitutively expressed in cancer cells, they can continue to survive even with little oxygen, contributing to the Warburg effect (38). Another metabolite of interest to the scientific community as it relates to cancer biology is the enzyme Isocitrate Dehydrogenase or IDH.

IDH is an enzyme of the TCA cycle (Figure 1-2) that catalyzes the oxidative decarboxylation of isocitrate to the metabolite α-ketoglutarate which, due to the high change in negative free energy associated with this reaction, has been termed an irreversible step of the TCA cycle and is very tightly regulated (2). Three different isoforms of this enzyme exist: IDH1, IDH2 and IDH3 which are all encoded by separate genes found in the nucleus. IDH3 is composed of two alpha chains, one beta chain and one gamma chain while both IDH1 and IDH2 are homodimers (70). While all three isoforms catalyze the same reaction, IDH1 and IDH2 are able to reverse this process if need be and help to regulate the amount of α-ketoglutarate and NADPH pools produced within the cell (47). The isoforms are localized to different subcellular compartments in the cell, with both IDH2 and IDH3 contained in the mitochondria while IDH1 is found in the cytosol and in peroxisomes. Both IDH1 and IDH2 utilize the electron acceptor $NADP^+$ while IDH3 uses NAD^+ as an electron acceptor to generate NADPH and NADH respectively. This reaction requires the use of a Mn^{2+} ion found in the active site of the enzyme which helps to stabilize the isocitrate molecule as it is converted to α-ketoglutarate through a highly unstable enol intermediate (Lehninger, 2008). This reaction utilizes a molecule of

$NAD(P)^+$ which oxidizes a hydride transfer to create one molecule of NAD(P)H which can be used in the oxidative phosphorylation pathway found in the mitochondria. This oxidation of isocitrate creates the oxalosuccinate intermediate which remains in the active site of the enzyme where one molecule of CO_2 is released. With the help of the Mn^{2+} ion, the enol intermediate is stabilized and converted into α-KG which can be used elsewhere in the cell. α-KG is an essential molecule of the TCA cycle and is required for many enzymes in relation to metabolism including those needed for amino acid synthesis, nitrogen transport, collagen formation and oxidation reactions among others. Of particularly interesting importance is the fact that α-KG is integral to many enzymes including a family of dioxygenases called the Ten Eleven Translocation (TET) family. The effect this enzyme has on the cell is quite intriguing and was examined in this thesis to discern if the alterations caused by IDH reached into the mitochondrial genome where cellular metabolism is regulated.

The Role of Isocitrate Dehydrogenase in Gene Expression and Cellular Differentiation

The TET family of dioxygenases is composed of three isoforms: TET1, TET2, and TET3 which utilize α-ketoglutarate, as well as Fe^{2+} and O_2 to catalyze the conversion of 5-methylcytosine to a novel base, 5-hydroxymethylcytosine (32). Little is known about this new DNA modification although recent research points to its possible role in DNA demethylation and ultimately gene re-expression (Lu and Thompson, 2012 and 24). The TET enzymes initially oxidize the 5-methylcytosine base to 5-hydroxymethylcytosine and further oxidize this alteration to 5-formylcytosine and finally 5-carboxylcytosine where it is rapidly removed from the cell by the enzyme Thymine DNA Glycosylase (TDG) (33,26). The TDG enzyme recognizes the 5caC residue as a mismatch to the usual guanosine and replaces the modified cytosine with a newly unmodified cytosine residue via the base excision repair machinery. It is through this mechanism

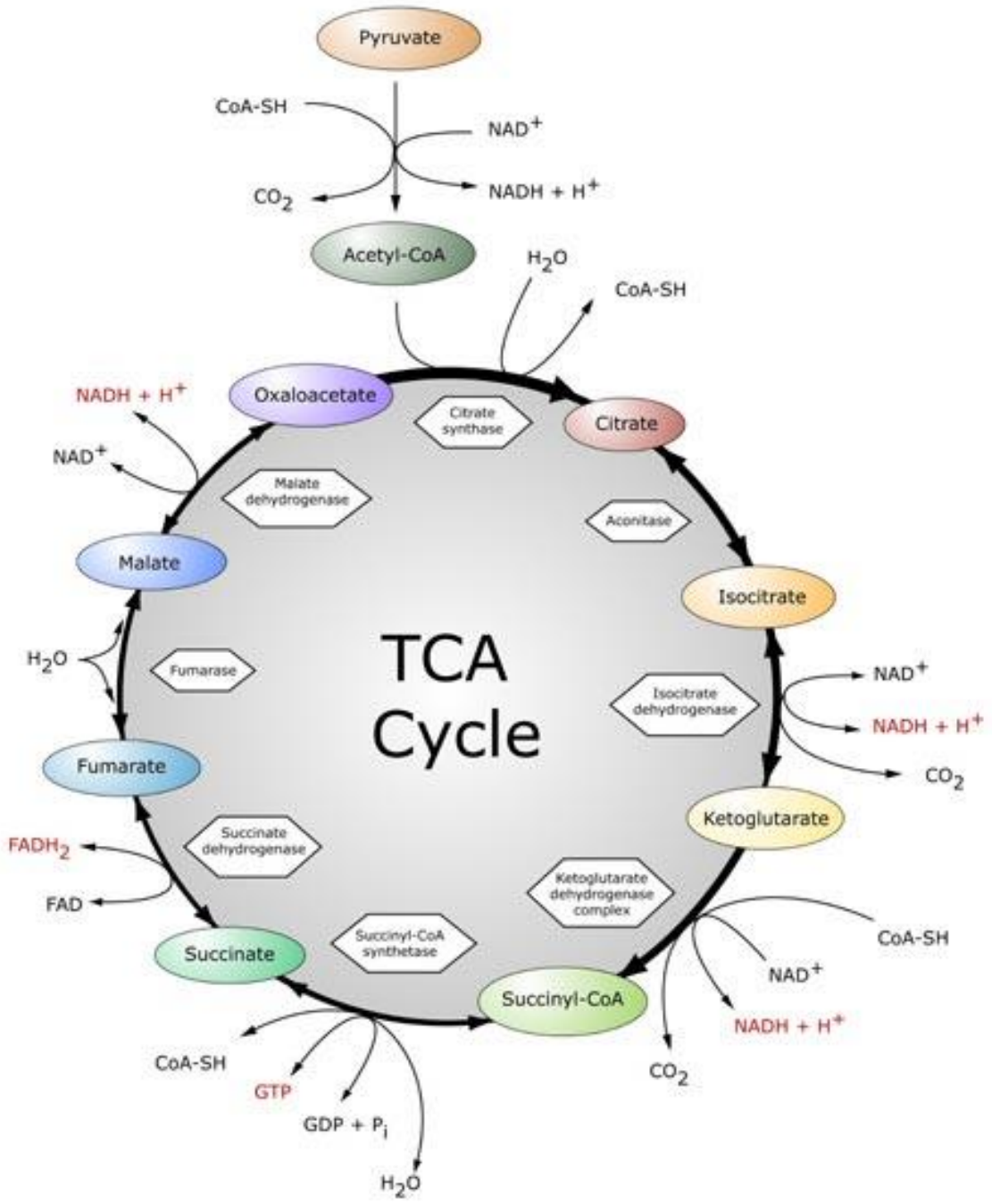

Figure 1-2: **The Tricarboxylic Acid Cycle**. Enzymes are boxed in hexagonal shapes while products of enzymatic reactions appear in colored ovals. By-products of each reaction are also displayed.

that DNA demethylation is possible which ultimately leads to the re-expression of genes that were silenced by methylation of the cytosine residues (85). Reports have uncovered that more than 70% of grade II and grade III gliomas, most secondary glioblastomas and even some instances of acute myeloid leukemia contain a somatic point mutation in the *IDH1* or *IDH2* genes (87, 50), and this has been shown to lead to the distinctive CpG island methylator phenotype (CIMP) which is characterized by a global hypermethylation of the genome and defines a distinctive subgroup of gliomas (56). Patients known to contain the CIMP phenotype have been diagnosed earlier on in age and usually present with a more favorable prognosis than CIMP negative patients (12 and 52). In 2012, research discovered that, not only are the IDH mutation and the CIMP phenotype connected, but IDH1 mutation is sufficient to establish this hypermethylator phenotype in the genome of many glioma samples by disrupting DNA and histone methylation patterns produced in the nucleus (76). This mutation occurs at analogous arginine residues found in the active site of the enzymes and is a defining feature of tumor progression (4). One mutation, R132H is found in the IDH1 isoform while the IDH2 isoform most commonly contracts the R140Q or R172K mutation in these cancerous instances. Once originally thought to lead to a loss of IDH function, these mutations result in the loss of stability of the β-carboxyl of the isocitrate metabolite which is docked in the enzyme (82). Studies investigating both IDH1, the cytosolic isoform, and IDH2, the mitochondrial isoform, have revealed these mutations are heterozygous and that one wild-type allele is typically maintained (15, 82). This heterozygosity was predicted to confer a novel gain of function on these enzymes, and a neomorphic activity was demonstrated (15) leading to the synthesis of a new metabolite, 2-hydroxyglutarate (2-HG), an analog of α-ketoglutarate. 2-HG contains a hydroxyl group at the carbon 2 position whereas the carbon 2 position of α-ketoglutarate has an oxygen molecule

bound to it. The D (-) enantiomer of 2-HG is typically produced via the IDH mutation. It is believed that while the wild-type allele of IDH is used to convert isocitrate into α-ketoglutarate, the mutant allele can use the newly synthesized metabolite, as well as NADPH, to reduce it to 2-HG and create a molecule of $NADP^+$ in the process. This new molecule, 2-HG, which is commonly kept at low levels below 0.1nM (47), builds up substantially within the cell and consequently reduces the pool of α-ketoglutarate available. Researchers have shown that the metabolite 2-HG competitively inhibits the active sites of enzymes, such as the TET dioxygenases mentioned above (86). This inhibition of the TET enzymes leads to a decrease in the amount of 5-hydroxymethylcytosine produced and a subsequent rise in 5-methylcytosine residues in the nuclear DNA of the cell which establishes a hypermethylator phenotype such as that described (76). Of particular note is the distribution of IDH mutations in cancer. In acute myeloid leukemia (AML), the IDH1 and IDH2 mutations are mutually exclusive to each other as well as to TET2 mutations commonly found in myeloid cancers (1, 41,16), confirming the hypothesis that these mutations have a similar mechanism of action to disrupt the methylation patterns of the cell (21). Along with the inhibition of TET enzymes, other α-ketoglutarate dependent enzymes are affected by this mutation including histone modifying enzymes which control cellular differentiation and gene expression. Experiments performed in this thesis were aimed at more fully understanding the far-reaching effects of mutant IDH.

The Human Mitochondrion and its Role

The circular double stranded mitochondrial genome is approximately 16.6 kilobases in length in humans and roughly 16.3 kilobases in mice (Figure 1-3). Each cell contains anywhere from 100 to 10000 copies of mitochondrial DNA (20) and these mitochondrial genomes lack introns. Each strand, designated as either the heavy strand (guanosine rich) or light strand (guanosine poor),

encodes 28 and 9 genes respectively which, when combined, account for 13 protein coding genes, 22 transfer RNAs and two ribosomal RNAs. The 13 open reading frames produce 13 proteins that are integral components of four of the five oxidative phosphorylation complexes necessary for energy production in the cell (79). The mitochondrial genome also contains a control region termed the D-loop where an origin of replication can be found as well as three separate transcriptional promoters. These three promoters each transcribe a specific polycistronic message from the mitochondrial genome. Heavy strand promoter 1 (HSP1) produces a message that encodes the two rRNAs, which are designated as 12S and 16S, and terminates at the end of the 16S gene. This termination process is dependent upon the mitochondrial transcription termination factor (mTERF) which creates a loop between the HSP1 promoter and the end of the 16S gene to control the level of H1 transcript present in the cell (51). HSP2 is close to the 5' end of the 12s rRNA gene and encodes the entire heavy strand. The light strand promoter (LSP) allows for the transcription and translation of the entire light strand of the mitochondrial genome. mtDNA transcription is initiated by the mitochondrial transcription factor A (TFAM), as well as many other factors, which work to unwind the DNA and allow for promoter recognition by the mitochondrial RNA polymerase (POLRMT) so that transcription can begin. Interestingly, mtDNA transcription functions independently of nuclear genomic transcription and can be initiated at different times in each mitochondrion found in the cell without any phase specificity meaning that not every mitochondrion is producing the same message at the same time (20). It is possible that this allows for greater production of necessary mitochondrial components when necessary in the cell.

Evidence suggests that mitochondria play an integral role in many disease states including Diabetes, Alzheimer's, Parkinson's disease and cancer, which has led many research

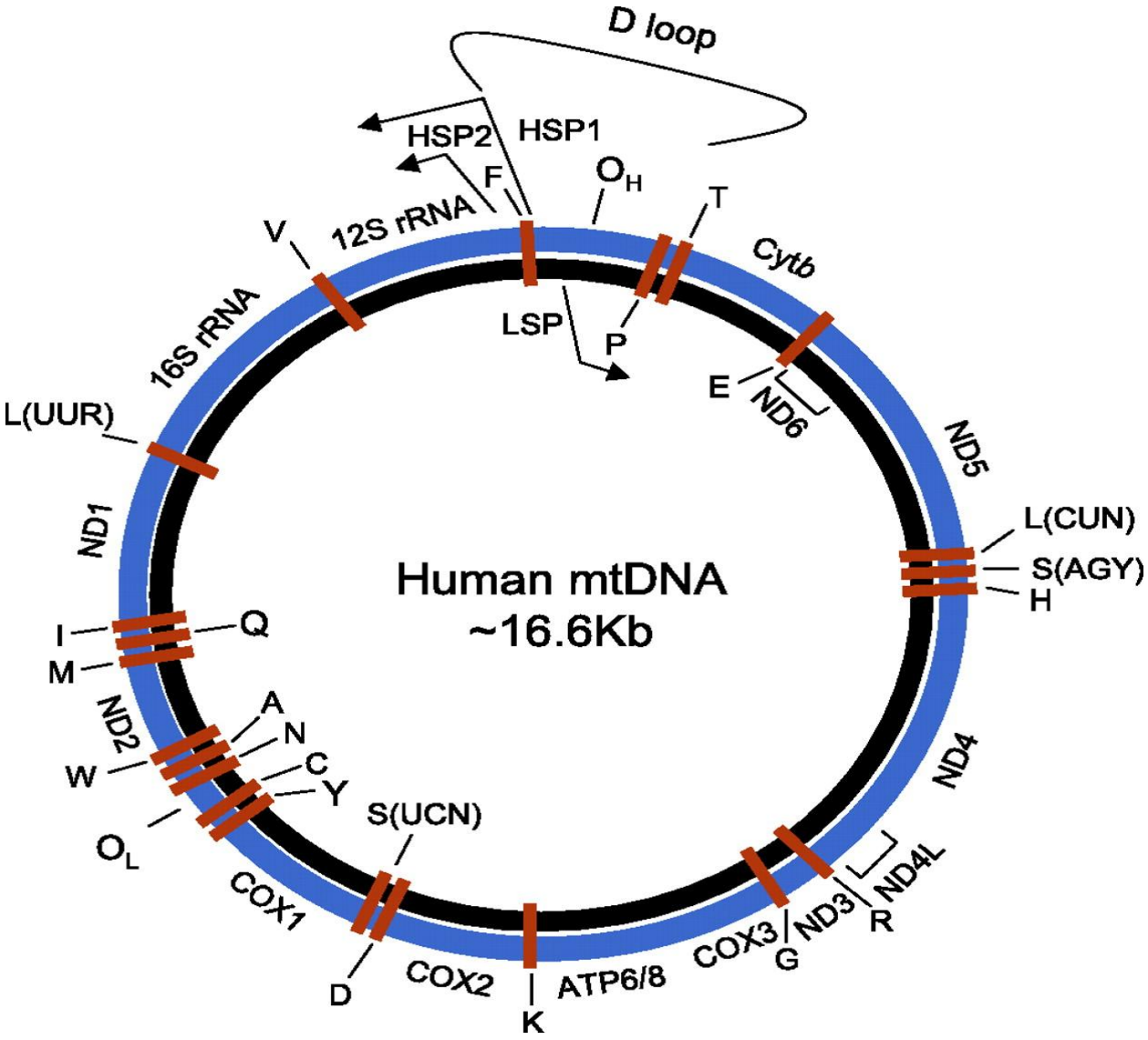

Figure 1-3: Organization of the mitochondrial genome in human cells. All genes encoded on this genome are shown as well as the start sites for the three different transcription products. The heavy strand is denoted in blue, the light strand denoted in black and the tRNA genes are denoted in red.

laboratories to investigate the effects of mitochondrial function and regulation in these illnesses (45, 66). Mitochondrial DNA is predominantly inherited via maternal inheritance in mammals although rare cases of paternal inheritance have been reported (Shapira, 2006). Most mitochondria in a cell contain the same genetic code, which is termed homoplasmy. However, due to the large volume of mitochondria in the cell and the generation of free oxygen radicals produced by the respiratory chain itself, mutations can arise much more frequently than in the nuclear genome. Also, during replication, different numbers of mitochondria can segregate into the new daughter cells produced. The coexistence of mutant and wild-type mtDNA is called heteroplasmy, and mutations accumulate as the individual organism ages (Shapira, 2006). Mitochondrial mutations commonly take the form of either a deletion or random point mutations. Many different mtDNA mutations have been characterized and result in debilitating pathologies (42). Not only are mtDNA mutations important in disease states, but mutations can also occur in the nuclear DNA that encodes mitochondrial proteins. Indeed, in Alzheimer's disease, the gene encoding the amyloid precursor protein is mutated which leads to the impairment of oxidative phosphorylation processes in the cell as well as severe accumulation of senile plaques, a common attribute of this disease (Lin, 2006). Transcription of mitochondrially encoded peptides, such as the ND6 and ND2 genes on the mitochondrial light and heavy strands respectively, have also been shown to be reduced by 50% in the brains of many Alzheimer's patients suggesting an effect on mitochondrial gene transcription (79).

Evidence also suggests that change in mitochondrial function contribute to the ageing process (14). As an organism ages, electron transport chain function declines leading to a subsequent increase in the production of reactive oxygen species (71). These changes seem to be brought on by the increasing levels of mutations found in the mitochondrial DNA over time as

oxidative phosphorylation processes are continuously being employed. The generation of ROS inside the cell leads to the activation of the mitochondrial permeability transition pore (mtPTP) which in turn increases the likelihood of apoptosis by releasing the contents of the mitochondria into the cellular cytosol, including the apoptotic promoting molecule, cytochrome c (77 and 79). Cellular ageing results in the decreased activity of mitochondrial encoded proteins such as those found in Complex I and Complex IV of the electron transport chain (71). These studies confirm the critical importance of the mitochondrial genome in cellular homeostasis. With this knowledge, this thesis aims to uncover how a mutant enzyme, IDH, can alter mitochondrial transcription and thereby alter this organelle's function.

The Warburg Hypothesis

In the 1920s, Dr. Otto Warburg hypothesized that cancer is a disease of metabolism and can be interpreted as a disease of mitochondrial misregulation (Presner, 2011). Specifically, Warburg proposed that in cancer, there is a general shift in how cells acquire nutrients, generate energy and regulate their metabolism (Figure 1-4). In the presence of oxygen, most non-proliferating undifferentiated cells use the mitochondrial tricarboxylic acid (TCA) cycle to metabolize glucose into carbon dioxide by oxidative phosphorylation (83). Through multiple enzymatic conversions and phosphorylations, glucose is broken down into two molecules of pyruvate, a three carbon chain, in the cytoplasm during glycolysis. These pyruvate molecules are then shuttled through the mitochondrial membrane and converted to acetyl-coA molecules where they undergo oxidation to create adenosine triphosphates. Once inside the matrix of the mitochondrion, one molecule of acetyl-coA is metabolized to ultimately produce three molecules of NADH, one molecule of $FADH_2$, one molecule of GTP or ATP and two molecules of CO_2. The CO_2 molecules are taken out of the cell and exhaled out of the body. The GTP/ATP

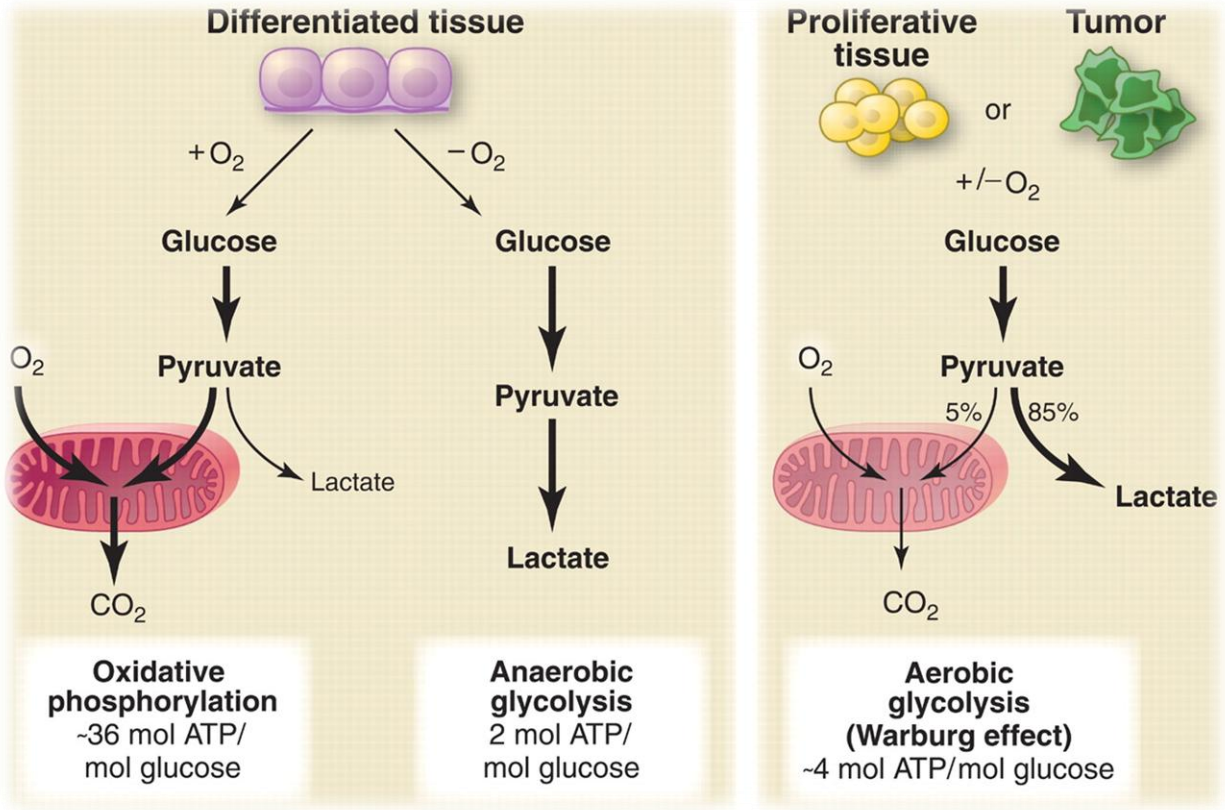

Figure 1-4: The Warburg effect. Comparing the difference in energy usage between differentiated tissues and proliferative or tumor tissues. The production of lactate is favored over oxidative phosphorylation in tumor samples whether in the presence or absence of oxygen. Figure adapted from Vander Heiden, 2009.

molecule generated can be used as a fuel source and energy producer. The NADH and $FADH_2$ molecules produced by the TCA cycle help to shuttle electrons through the mitochondrial inner membrane to O_2 molecules via oxidative phosphorylation. Through OXPHOS, H^+ ions are subsequently pushed into the intermembrane space to create a gradient of charge across the membrane. This gradient helps to provide the necessary energy to synthesize ATP from ADP and inorganic phosphate using the ATP synthase enzyme, which ultimately leads to the production of about 15 molecules of ATP which, when calculated, totals to about 32 molecules of ATP generated per molecule of glucose taken up in the diet (55). This vast amount of ATP is used as energy in the cell to do work and catabolize necessary materials.

In an environment where oxygen is in short supply, such as during strenuous exercise, mammalian cells will undergo glycolysis for quick ATP production. This process utilizes a glucose molecule to produce two molecules of pyruvate which are then reduced into a total of two molecules of lactate. Lactate can be used within the cell to generate two net molecules of ATP for energy. This production of ATP through glycolysis is much smaller than the amount of energy produced during oxidative phosphorylation and requires much more glucose to produce the same effect as the relatively small number of glucose molecules needed during aerobic conditions (46). Cancerous cells on the other hand, as observed by Warburg, tend to consume extremely large amounts of glucose and produce abnormally high levels of lactate even in the presence of ample supplies of oxygen (38). This process, termed aerobic glycolysis, has become a defining feature of most cancers of the human body. Today, Otto Warburg's hypothesis from more than 80 years ago has garnered much attention, as aberrant metabolism has evolved as another hallmark of cancer biology. In this present work, we aim to uncover how metabolism can

be altered by studying the mitochondrial genome and how the effects produced by a mutant enzyme can lead to dysregulation in this system.

The Study of Epigenetics as it Pertains to the Mitochondria

Early studies uncovered the existence of 5-methylcytosine residues in the human mitochondrial genome as well as in many other species including rat, hamster, mouse and cow. However, this idea of mitochondrial epigenetics has remained somewhat controversial over the past few years. Earlier reports point to the fact that mitochondrial DNA contains no histones and instead bundles its content into nucleoids tethered to the mitochondrial inner membrane by the protein TFAM (Transcription Factor A) along with other proteins necessary in mitochondrial maintenance (22). Also, there was no evidence to suggest that methylases can enter the mitochondria in mammals. The Taylor lab has addressed these problems and put this debate to rest once and for all. Recent evidence suggests that earlier assessments of the possibility of mitochondrial epigenetics may have been incorrect. DNMT1 has been shown to be able to enter the mitochondrial genome using an upstream start site in translation that encodes an N-terminal mitochondrial leader sequence, allowing mtDNMT1 to enter the mitochondria and methylate cytosine residues in the mitochondrial genome (68). The two *de novo* methyltransferases, DNMT3a (11) and DNMT3b (6), have also been suggested to enter the mitochondria, although this evidence is not as strong. While not much is known about this new mitochondrial DNA modification or its function, evidence has found that 5-methylcytosine preferentially exists at non-CpG dinucleotide sites as opposed to CpG dinucleotides which are more commonly found in nuclear DNA methylation patterns (Belizzi, 2013). The Taylor lab was also able to uncover the existence of 5-hydroxymethylcytosine residues in the mitochondrial genome; while the presence of this modification in the mitochondrial genome remains to be determined, levels in mouse

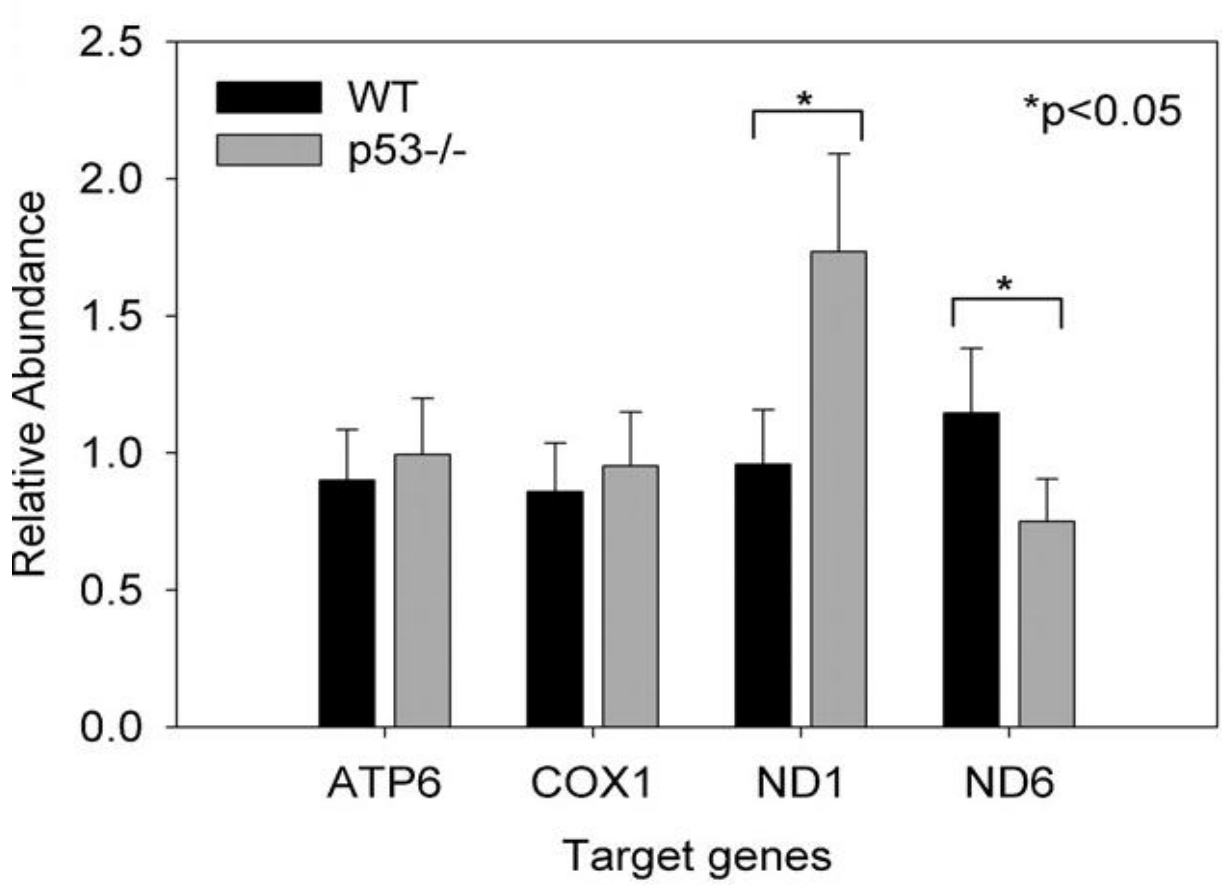

Figure 1-5: p53 status affects mitochondrial gene expression in a gene-specific manner. p53 affects expression level of the mitochondrial isoform of DNMT1 which can lead to an effect on gene expression of the protein-coding genes of the mitochondria. Levels of mRNA for ND1 and ND6 were differentially expressed after loss of p53. Adapted from Shock, 2011.

brain mitochondria have been implicated in the aging process (18). Also of note, cytosine modifications in the mitochondrial genome have a gene-specific effect on the protein-coding genes as ND6 (the protein encoded on the light strand) is downregulated while ND1 (the first protein-coding gene after the ribosomal RNA genes) is upregulated in p53-null cells which transcribe an abundance of mtDNMT1 mRNA (Figure 1-5 adapted from 68).With this finding, research has been performed to determine that the TET enzymes, as well as DNMTs, can cross into the mitochondria and perform their enzymatic functions on mtDNA (6). It stands to reason that while not much is known about mitochondrial epigenetics, mitochondrial methylation and its subsequent hydroxymethylation may have a serious effect on oxidative phosphorylation and energy production in the cell through their effects on gene transcription of the mitochondrial genome.

As mitochondrial dysfunction and metabolic reprogramming seem to play an important role in many disease states including cancer, it seems logical that mutant IDH protein would have a significant effect on mitochondrial epigenetic levels. Previous studies have identified the effect IDH mutation has on nuclear epigenetics as well as histone modifications to control gene expression and cellular differentiation respectively, but studies on a possible mitochondrial effect have been lacking at this time. We propose that IDH mutation would affect the levels 5-hydroxymethylcytosine residues found in the mitochondrial genome via production of 2-hydroxyglutarate which will lead to the inhibition of TET enzymes located within the mitochondria. A fall in 5-hydroxymethylcytosine content could lead to the establishment of a hypermethylator phenotype in the mitochondria as previously shown in the nuclear genome. To determine if any effects on mitochondrial epigenetics result when IDH mutations are introduced, an innovative technique, Methylated DNA Immunoprecipitation (MeDIP), will be used to

quantitate the 5mC and 5hmC residues found in the mitochondrial genome, as traditional Bisulfite sequencing cannot distinguish between these two modified bases (29). Quantitative polymerase chain reaction will be used to quantitatively determine how the levels of 5mC and 5hmC are affected in the mitochondrial genome, specifically the regions encoding the 12S and 16S rRNA genes.

Chapter 2: Materials and Methods

Cells, Cell culture, and plasmids used to determine the effect of IDH mutations on mitochondria

The cDNAs encoding IDH1, IDH2, L2HGDH or D2HGDH were obtained from the Arizona State University DNA repository and recloned into either pDEST26 C-FLAG (IDH) or pcDNA 6.2/myc (HGDH). Site directed mutagenesis was carried out using the Quick-Change Mutagenesis kit from Agilent to generate the IDH1 R132H and IDH2 R172K mutations. All cloning and mutagenesis was carried out by the Biological Macromolecule Core Laboratory at Virginia Commonwealth University. All plasmids were verified by DNA sequence analysis. Both HEK293 and HCT-116 cell lines were used in this project. Cells were grown in either 6-well plates (for protein extracts) or 100mm dishes (for DNA purification) in DMEM media with 10% Fetal Bovine Serum (Atlanta) in 10% CO_2 in a humidified incubator at 37°C.

Transient transfection

Once cells reached 70-80% confluency in their respective dishes, transient transfections were performed. Plasmids encoding epitope-tagged proteins are shown in Table 1. For 6-well plates, 2µg of each plasmid was diluted with 50uL of serum free DMEM media (high glucose) and 6µL Polyjet Transfection reagent (SignaGen Laboratories Cat #SL100688) was also diluted with 50µL serum free DMEM and added to diluted plasmid. After 15 minutes at room temperature, each transfection mixture was pipetted into a well containing 70-80% confluent cells. The cells were incubated overnight and the next day fed with DMEM media containing 10% FBS and 1% Penicillin-Streptomycin. Media was replaced 24 hours post-transfection and, after 48 hours, cells were harvested. Cells were washed twice with cold non-sterile PBS

Table 1: Table of expression plasmids used in this thesis. Antibobies needed for immunoblotting as well as antibiotics necessary for generation of stables is provided.

Sample	Plasmid 1	Plasmid 2	Western Blot Antibody Needed	Drug Selection for Stables
1	IDH1 wild-type		FLAG	Neomycin
2	IDH1 wild-type	L2HGDH	FLAG/Myc	Neomycin/Blasticidin
3	IDH1 wild-type	D2HGDH	FLAG/Myc	Neomycin/Blasticidin
4	IDH1 mutant		FLAG	Neomycin
5	IDH1 mutant	L2HGDH	FLAG/Myc	Neomycin/Blasticidin
6	IDH1 mutant	D2HGDH	FLAG/Myc	Neomycin/Blasticidin
7	IDH2 wild-type		FLAG	Neomycin
8	IDH2 wild-type	L2HGDH	FLAG/Myc	Neomycin/Blasticidin
9	IDH2 wild-type	D2HGDH	FLAG/Myc	Neomycin/Blasticidin
10	IDH2 mutant		FLAG	Neomycin
11	IDH2 mutant	L2HGDH	FLAG/Myc	Neomycin/Blasticidin
12	IDH2 mutant	D2HGDH	FLAG/Myc	Neomycin/Blasticidin
13	L2HGDH		Myc	Blasticidin
14	D2HGDH		Myc	Blasticidin
15	pIRES neo2			Neomycin
16	pcDNA3.1 myc/hisB			Blasticidin
17	Untransfected			
18	pMAX GFP			

(pH 7.4), scraped from dish and collected by centrifugation in pre-weighed 1.5mL centrifuge tubes. Media was removed and cold non-sterile PBS (pH 7.4) was used to wash each well twice. Cells were resuspended in 5μL/mg wet weight in SDS lysis buffer (2.25 mL EDTA free protease inhibitor tablets (Roche) resuspended in 2X sample buffer (62.5 mM Tris-HCl, pH 7.5, 5% glycerol, 2% SDS), 2.025mL water, 225 μL β-Mercaptoethanol) and sonicated on high power for 7.5 minutes at 30 seconds on, 30 seconds off intervals in a Diagenode Bioruptor bath sonicator and stored at -80°C for use in immunoblotting.

Samples for DNA extraction were transfected in 100mm dishes using 8μg of each plasmid, 20μL of Polyjet Transfection reagent and 150μL of serum free DMEM as diluents as described above for 6-well plates. After 15 minutes at room temperature, each transfection mixture was pipetted onto a plate containing 70-80% confluent cells. As previously described, media was replaced 24 hours post-transfection and at 48 hours post-transfection, cells were scraped into 1.5mL centrifuge tubes, pelleted and stored at -80°C for later DNA extraction.

Selection of Stable Transfectants

To create cell lines that stably express the plasmids of interest, either 100mm (Blasticidin resistance plasmids) or 150mm dishes (Neomycin resistance plasmids) were used. Cells were brought to 70-80% confluence and transfections were performed as described for transient transfections, above. For 150mm dishes, 300uL of serum free DMEM media, 12ug of each plasmid DNA and 30uL of polyjet reagent were used. Stable integration of plasmid was selected using neomycin (1mg/mL), blasticidin (2μg/mL) or both drugs together, as indicated in Table 1. An untransfected control was also used to ensure that plasmids were integrated into the cellular genome. Once colonies began to form on the dishes, 12 were removed using sterile

polyester Q-tips and placed in one well of a 12-well plate. Media was replaced with designated drug until colonies reached confluency in their wells. The cells were then trypsinized from their wells, pelleted and aliquoted into 100mm dishes (DNA isolation), 60mm dishes (protein extraction and immunoblot) or individual wells of a 24 well plate for cryopreservation, using freezing media (90% FBS, 10% DMSO). 300μL of total cells were plated for freezing, while 1/3 total volume of cells were aliquoted into 60mm dishes and the remaining cells were aliquoted into 100mm dishes.

Protein Concentration Assays

The Bradford Assay was used to determine protein concentration of each sample that was harvested from either 6-well plates (transient transfection) or 60mm dishes (stable transfection). Duplicate assays were conducted for each sample as well as for the standard curve. Standard curve samples contained 699-799μL of H_2O at 20μL intervals, 1μL of SDS lysis buffer and BSA (0-10μg) while sample tubes contained 799μL of H_2O and 1μL of protein extract. 200μL of BioRad dye concentrate (Cat # 500-0006) was added to each tube. After a 15 minute incubation at room temperature, absorbance was determined using a spectrophotometer at 595nm wavelength.

Immunoblot Assays of Protein Expression

Each sample (40μg) was mixed with an equal volume of Laemmli Sample Buffer (475uL Laemmli Buffer (Cat# 161-0737)/25uL of β-mercaptoethanol), and total volume was adjusted to 37μL with SDS lysis buffer. Samples were run alongside Precision plus Dual Color protein ladder (Biorad Cat# 1610374) in 1X Running Buffer (25mM Tris, 250mM glycine, 0.1%

Table 2: List of antibodies used for western blotting

Antigen	Primary Ab obtained from	Primary Ab Dilution	Blocking Buffer	Secondary Ab Used	Secondary Ab Dilution	Expected protein band size	Chemiluminescent substrate prepared
FLAG	Sigma Aldrich Cat. F1804	1:500	Starting Block	Goat Anti-Mouse	1:15000	50 kD	½ Dura
Myc	Invitrogen Cat. R950-25	1:2000	Starting Block	Goat Anti-Rabbit	1:10000	50 kD	Full Pico
IDH	Cell Signaling Cat. 12652	1:1000	Starting Block	Goat Anti-Rabbit	1:1000	50 kD	¼ Dura
VDAC	Cell Signaling Technology Cat. 4661	1:2500	Starting Block	Goat Anti-Rabbit	1:3000	32 kD	Full Pico
Cyclophilin A	Millipore Cat. 07-313	1:5000	Starting Block	Goat Anti-Rabbit	1:5000	18 kD	Full Pico

SDS). Samples were boiled for 10minutes. Proteins were resolved on a 10% polyacrylamide gel for one hour at 150 volts. Gel was washed in water then transferred to a PVDF membrane (Millipore, Cat# IPVH0010) that was pre-washed in 100% Methanol. Transfer was performed in ice bath with stir bar placed in transfer apparatus for 50 minutes at 100 volts in 1X Transfer Buffer (25mM Tris-HCl, pH 8.3, 192mM glycine, 10% Methanol and 0.04% SDS), and membranes were incubated in Starting Block buffer (Thermo Scientific) overnight at 4^{o}C. Membranes were washed in 1X TBS-T (25mM Tris-HCl, pH 8.3, 192mM glycine, 0.04% SDS, 0.1% Tween-20) for 5 minutes and exposed to epitope-specific primary antibodies for one hour. All primary and secondary antibodies, as well as dilutions used, are listed in Table 2. VDAC or Cyclophilin A was used as a loading control. After one hour incubation, membranes were washed in 1X TBS-T thrice for 5 minutes and then incubated with secondary antibody for one hour (using antibodies specific for Fc regions of primary antibodies). After incubation, three 10 minute washes in 1X TBS-T were performed. During this time, chemiluminescent substrates were prepared using either West Dura (Pierce, Cat# 34075) or West Pico (Pierce, Cat# 34080). Once washed, membranes were dried, incubated in substrates for 3 minutes, wrapped in saran wrap and exposed on film to determine protein expression levels.

Purification of DNA from transfected cell lines

Once protein expression levels had been determined, cell pellets from 100mm dishes for DNA extraction were lysed using the DNeasy Blood and Tissue Kit from Qiagen (Cat# 69504), following the manufacturer's protocol. An RNase treatment was used to ensure pure DNA was collected and any RNA was destroyed in each sample, since RNA can also be modified at cytosine residues. The DNA was further purified to destroy any remaining RNA by incubating with 0.5M NaOH at 37^{o}C overnight in a water bath. DNA was recovered following phenol-

chloroform extraction by ethanol precipitation. Extracted DNA was resuspended in 200uL of AE buffer (Qiagen) and concentration was measured on the NanoDrop spectrophotometer to determine DNA concentration. With this purified DNA, samples were sonicated twice at high power for 15 minutes using intervals of 30 seconds on, 30 seconds off to obtain DNA fragments between 200-700bp. This sonicated DNA was resolved on a 1.5% agarose gel for 70 minutes at 100 volts to verify the correct fragment size of DNA needed for MeDIP experiments.

MeDIP/Hydroxy-MeDIP Assay

Once DNA of correct length and purity was obtained, MeDIP was performed to obtain methylated and hydroxymethylated DNA of sample (visual of MeDIP displayed in Figure 2-1). As the 5-methylcytosine and 5-hydroxymethylcytosine antibodies were produced in mouse and rabbit species respectively, both an IgG mouse and IgG rabbit antibody were necessary as measures of baseline in this experiment. 4ug of purified and sonicated DNA was brought up in Tris EDTA buffer (10mM Tris, 1mM EDTA) to 450uL total volume. This procedure was repeated to generate 4 tubes of 4µg for each DNA sample used in MeDIP experiment. These tubes were subjected to denaturation by submerging in boiling water for 10 minutes and then immediately placed on ice for 10 minutes. After denaturation, samples were mixed with 51uL of 10X IP buffer (100mM Na-Phosphate pH 7.0, 1.4M NaCl, 0.5% Triton X-100) and rotated end-over-end at 4°C overnight with 2µg of one specific antibody for each tube which include: IgG rabbit monoclonal control (Santa Cruz Biotechnologies, Cat# SC-2027), IgG mouse polyclonal control (Millipore, Cat# 12-371), 5-methylcytidine antibody (Epigenetek, Cat# A-1014) or 5-hydroxymethylcytidine antibody (Active Motif, Cat# 39769). To ensure proper capture of 5-methyl and 5-hydroxymethyl residues, IgG protein beads were pre-blocked prior to use in immunoprecipitation the following day. Beads were washed three times in 0.1% PBS-BSA

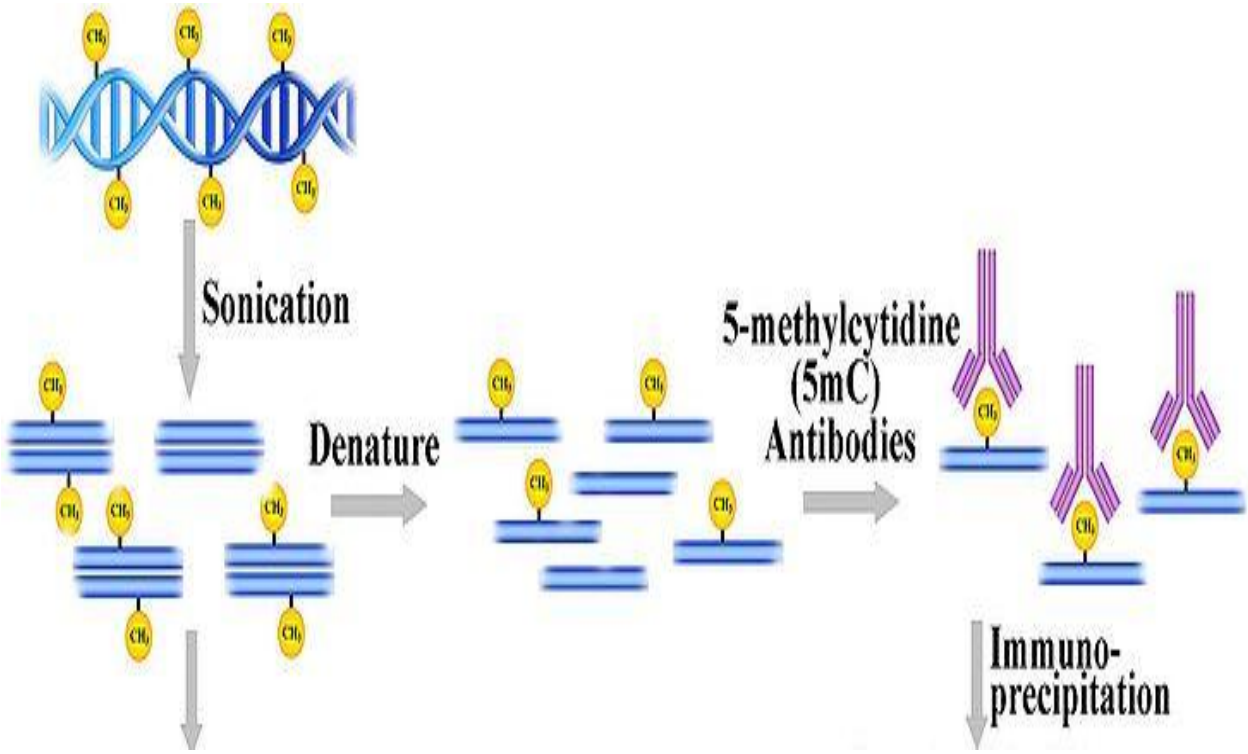

Figure 2-1: Schematic representing the protocol used for the MeDIP/Hydroxy-MeDIP method. Protocol followed to ensure adequate isolation of methylated/hydroxymethylated DNA residues respectively.

(10mg of BSA were dissolved in 9mL of 1X PBS pH7.4 and 1mL of H_2O), each time being rotated end-over-end at 4°C for 5 minutes and pelleted at 4500rpm for 5 minutes at 4°C. These beads were resuspended in an equivalent volume of 1X IP buffer and blocked using 5µg/30µL IgG bead slurry BSA and 5µg/30µL IgG bead slurry sonicated Lambda DNA. Beads were rotated at 4°C for three hour incubation. Afterwards, beads were washed three more times with 0.1% PBS-BSA, rotated and pelleted as before. 50% IgG bead-1X IP slurry was prepared again and 20µL of slurry was aliquoted into each sample to capture antibodies that adhered to DNA regions of interest and rotated with beads for 2 hours at 4°C. After incubation, beads were pelleted and supernatants of IgG control samples were saved for later use as input samples for quantitative PCR. Beads of all samples were then washed with 500µL 1X IP solution, rotated end-over-end for 10 minutes and pelleted at 4500rpm for 2 minutes at 4°C three times. After pelleting beads for the third time, samples were resuspended in 250µL Proteinase K digestion buffer (50mM Tris pH 8.0, 10mM EDTA, 0.5% SDS) and 3.5µL of Proteinase K (20mg/mL) was added to each sample and tubes were left to incubate for 3 hours at 50°C in a rotating water bath. Samples, including saved supernatants from IgG controls, were then extracted with of 25:24:1 phenol:chloroform:isoamyl alcohol (250µL), spun at 13200rpm for 5 minutes at 4°C and the aqueous layer containing DNA was removed. Samples were then extracted with and an equivalent volume of chloroform (250µL), and spun again at 13200rpm for 5 minutes at 4°C. The top aqueous layer was collected and precipitated with $1/10^{th}$ the volume of 3M sodium acetate (25µL), glycogen (4µL) and 100% ethanol (750µL) to recover immunoprecipitated DNA. Samples were left to precipitate overnight at -80°C. The following day, samples were spun at 13200rpm for 20 minutes at 4°C to pellet DNA. Supernatant was removed and 70% Ethanol (500µL) was dispensed into each sample and spun again at 13200rpm for 10 minutes at 4°C.

Supernatant was removed from pelleted DNA and samples were dried for 10 minutes, resuspended in 75µL TE buffer and stored at -20°C for later use. For the experiment using HCT116 cells, the supernatants of each IgG control were not collected and the material that bound to the beads in the IgG mouse or IgG rabbit samples was used as a baseline to compare to their respective 5-methylcytosine or 5-hydroxymethylcytosine antibodies. This experiment was presented as a measure of the fold change difference between the IgG controls and their respective 5mC and 5hmC counterparts using the following equation:

$$\text{Fold Change} = 2^{-\Delta\Delta C(t)}$$

where ΔΔC(t) is the value obtained by subtracting the IgG cycle threshold values from the experimental (5mC or 5hmC) cycle threshold value after normalization to the H_2O blank cycle threshold values. For experiments involving HEK293 cells, supernatants from immunoprecipitated IgG controls were collected and used to quantify amount of input DNA using qPCR.

Assessment of DNA-IPs

Endpoint PCR was performed to determine a visual difference between both 5mC and 5OH and their IgG controls. Primers used were for both the 12S and 16S regions of the mitochondrial genome:

12S-Forward: 5′-AGTTCACCCTCTAAATCACCACG-3′

12S-Reverse: 5′-TGACTTGGGTTAATCGTGTGACC-3′

16S-Forward: 5′-ACCTTACTACCAGACAACCTTAGCC-3′

16S-Reverse: 5′-TAGCTGTTCTTAGGTAGCTCGTCTGG-3′

12S and 16S master mixes were produced using 12.5µL of HotStart Taq DNA polymerase, 1µL of 10µM forward and reverse primers and 8.5µL H_2O per tube. 24µL of Master Mix, along with 1µL of sample DNA was placed into clear microfuge tubes while PCR was run at 35 cycles under the following cycling conditions:

1. 95^0C for 15 minutes
2. 94^0C for 30 seconds
3. 55^0C for 30 seconds
4. 72^0C for 30 seconds
5. Go to Step 2 35 times
6. 72^0C for 5 minutes
7. 4^0C forever

H_2O blank as well as input DNA from the supernatant collected during the MeDIP protocol was used as both a negative and positive control respectively. PCR amplified products were then resolved on a 1% agarose gel for 70 minutes at 100 volts.

Quantitative PCR measurement of mtDNA in DNA-IP samples

Quantitative PCR was carried out using the same primers and cycling conditions used for Endpoint PCR. Samples were amplified over 35 cycles using Quantitect Sybr Green Dye HotStart Taq mastermix from Qiagen. The following cycling conditions were used: A no template control was run to detect any background amplification, while a standard curve was produced using 10-fold serial dilutions to 1:1000 of the wild-type IDH1 IgG mouse control sample following IP. Both IgGM and IgGR inputs were diluted 1:100 while samples treated with the 5OH antibody were diluted 1:5. Dilution factors for each treatment of each sample were

measured using the Opticon software and this data was expressed as a percentage of the input. Furthermore, data was normalized to IDH 1 or IDH2 wild-type to determine the difference in abundance of each epigenetic modification.

Treatment with Octyl-(D)-2-hydroxyglutarate

HEK293 cells were plated in 100mm dishes at 30-40% confluency and fed using 10% FBS DMEM. Cells were left to incubate overnight in 10% CO_2 incubator at 37°C. The following day, cells were treated with either 1µL or 10µL of 10mM stock solution of octyl-(D)-2-hydroxyglutarate (Toronto Research Chemicals Inc.) in DMSO to bring concentrations to 1µM or 10µM respectively. A no-drug control was used in this experiment as well as a DMSO vector control. Drug was left on plates for 48 hours and media was not removed. Cells were scraped from dish after 48 hours and pelleted for DNA isolation. The DNeasy Blood and Tissue Kit from Qiagen was used as previously described and samples were sonicated twice at high power for 15 minutes using intervals of 30 seconds on, 30 seconds off to obtain fragments of DNA between 200 and 500 base pairs. Sonicated samples were resolved on a 1% agarose gel for 70 minutes at 100 volts. MeDIP technique was followed as described above to isolate 5-methyl and 5-hydroxymethyl residues found in cells. 12S and 16S rRNA regions of the mitochondrial genome were amplified using both Endpoint and qPCR as described to determine the effect of this cell permeable analog of 2-hydroxyglutarate on the mitochondrial epigenome. Conditions for PCR were followed as described above.

Treatment with an IDH1 R132H Mutation Inhibitor, AGI5198

HEK293 cells that stably express the IDH1 R132H mutation were plated in 100mm dishes at 30-40% confluency and fed using 10% FBS DMEM. Cells were left to incubate

overnight in 10% CO_2 incubator at 37°C. The following day, cells were treated with 5µL of 10mM stock solution of AGI-5198 drug, a known IDH1 R132H mutation inhibitor, bringing the concentration of drug to 5µM. This stock solution was initially resuspended in DMSO. Both a no-drug treated and DMSO control was used for this experiment. After drug treatment, cells were harvested 48 and 144 hours later. Media was replaced 72 hours after initial treatment for 144 hours treated cell. Cells were scraped and pelleted for DNA isolation using the Qiagen Tissue and Blood kit as previously described. Samples were sonicated and subjected to MeDIP protocol as detailed above. To determine if this inhibitor drug had any effect on the mitochondrial epigenome, the 12S and 16S primer pairs (listed above) were amplified using both Endpoint and qPCR for a visual as well as quantitative determination.

Chapter 3: Results

The effect of IDH mutations on mitochondrial epigenetics using the HCT-116 cell line

Preliminary studies were performed using HCT116 cells to determine if the IDH1 R132H or IDH2 R172K mutations affect the levels of 5-methylcytosine and 5-hydroxymethylcytosine found within the mitochondrial genome of this cell line. These studies were originally begun in the summer of 2012 by Hannah Gardner. HCT-116 cells were used in these studies because they have an abundance of mitochondria within each cell. Cells were transfected with the plasmids shown in Table 1 to determine how either the cytoplasmic (IDH1) or mitochondrial (IDH2) isoforms of IDH would affect the epigenome of the mitochondria. Co-transfection of IDH mutant samples with the (D)-isoform of 2-HGDH was also carried out to determine whether changes in the level of 5-hydroxymethylcytosine resulting from effects of mutant IDH could be rescued, since this enzyme, under normal conditions, converts 2-hydroxyglutarate to α-ketoglutarate to keep the levels of 2-HGlow within the cell. The 2-HGDH enzyme should therefore relieve the competitive inhibition of the TET enzymes by 2-HG. A GFP control was used to visualize the efficiency of transfection in this cell line.

Cells transfected with GFP were viewed under a fluorescence microscope 24 and 48 hours post transfection as a measure of transfection efficiency (Figure 3-1). After 24 post transfection, 30-40% of cells glowed green under the microscope. 48 hours post-transfection, approximately 75% of cells glowed green under the fluorescent microscope indicating the level of transfection efficiency. After 48 hours, transfected cells were harvested for protein expression analysis from the transfected plasmids. Once the protein concentration of each sample was determined, immunoblotting was performed to determine protein expression level of each

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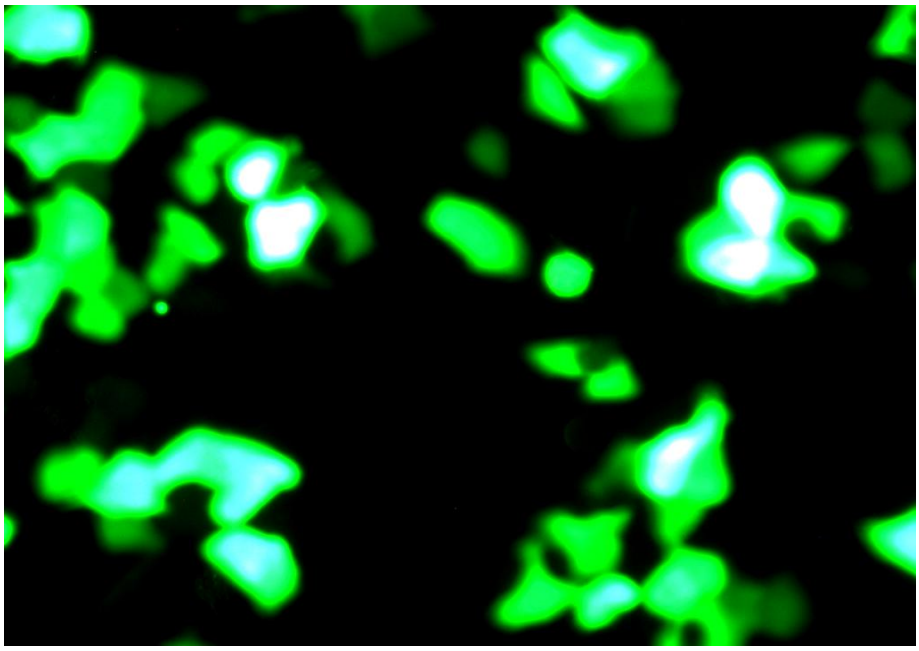

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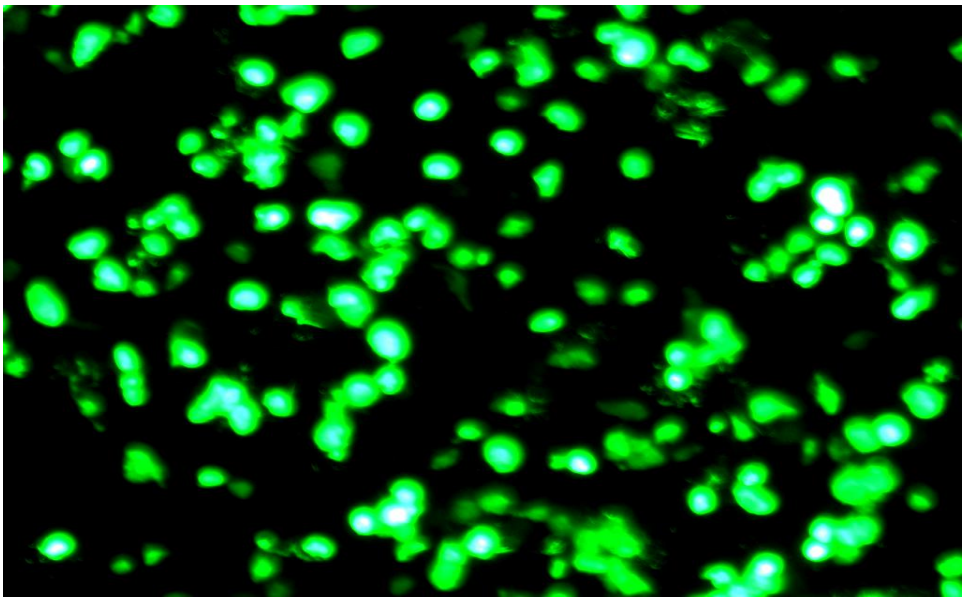

Figure 3-1: Determining transfection efficiency in HCT-116 cell line. Microscopy images of GFP transfected HCT-116 cells 24 hours (A) and 48 hours (B) after initial transfection to determine efficacy of transient transfection

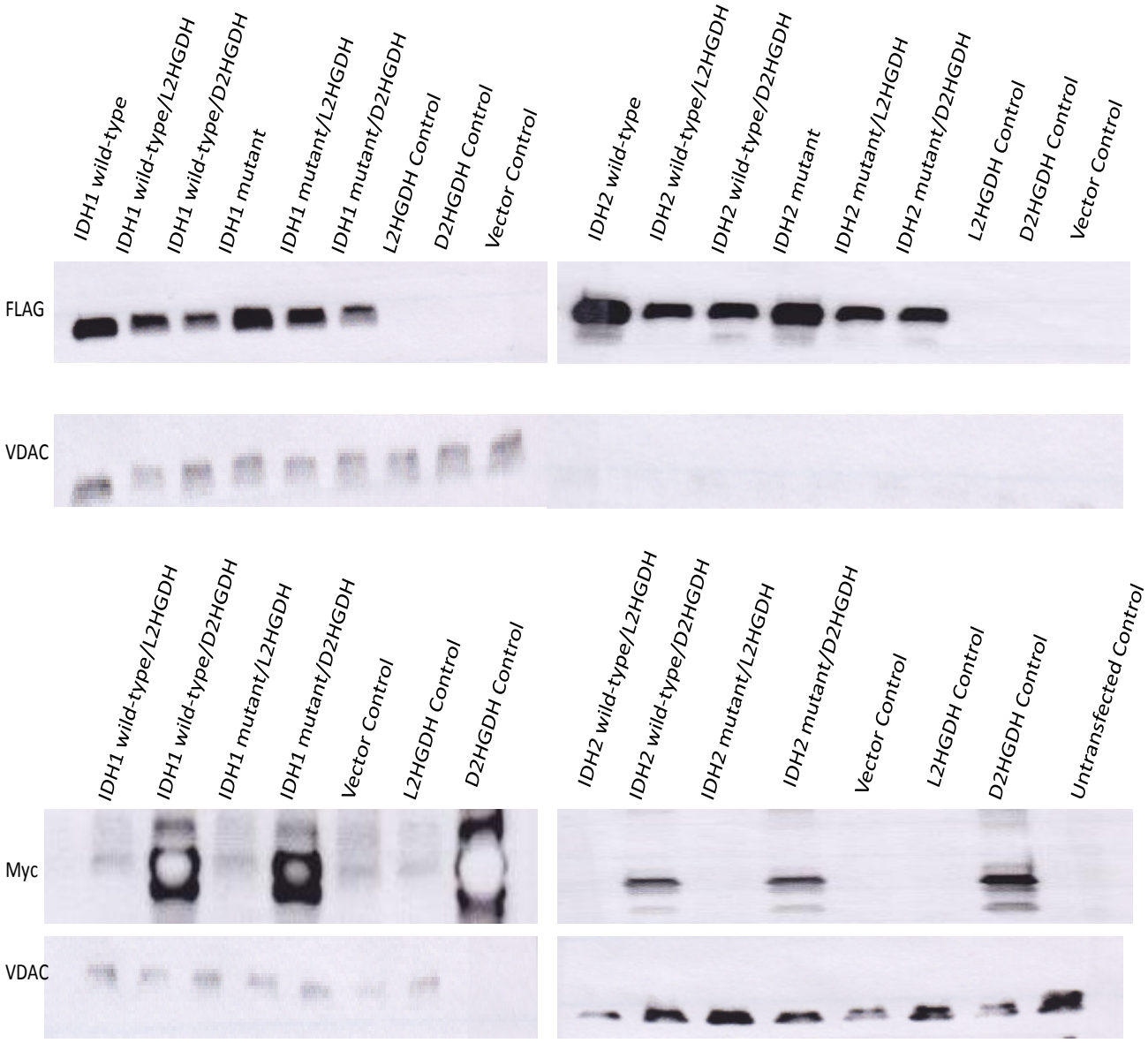

Figure 3-2: Analysis of protein expression levels of transiently transfected HCT-116 cells using immunoblotting. IDH proteins were analyzed using a FLAG-specific antibody while 2HGDH proteins were analyzed using a Myc antibody. VDAC was used as a loading control. These immunoblots were performed by Hannah Gardner.

sample. FLAG-tagged IDH samples were examined using an antibody specific to the FLAG tag located at the C-terminus of each IDH protein. A MYC antibody was used to visualize the MYC-tagged 2-hydroxyglutarate dehydrogenase plasmids that were co-transfected into the appropriate samples. A VDAC antibody was used to ensure equivalent loading of each sample onto the gel as it is a common ion channel found on the surface of the outer mitochondrial membrane. As can be seen in Figure 3-2, each sample expressed the appropriate IDH plasmid while co-transfected samples expressed both FLAG-tagged IDH as well as the MYC-tagged (D)-2HGDH plasmid. Samples transfected with the L-isoform of 2HGDH did not appear to express the epitope tag encoded on its plasmid, although DNA sequence analysis verified the correct in-frame cloning of this cDNA.

After expression levels were confirmed, the cells pelleted for DNA extraction were thawed and total cellular DNA, which includes mitochondrial DNA, was isolated from each sample. These samples were sonicated, to shear the DNA to a length of 200-500 base pairs to avoid amplifying modified DNA outside the regions of interest. The appropriate sonication of each sample was confirmed using a 1% agarose gel stained with Ethidium Bromide. Once the proper length of DNA was obtained for each sample, MeDIP was performed as detailed in the methods section. After MeDIP, endpoint PCR was utilized at 35 cycles to obtain a visual representation of how much modified DNA was present in each sample compared to its specific IgG control (Figure 3-3). The signal for the 5-hydroxymethylcytosine residues found in the 12S and 16S regions of the mitochondrial genome is very robust and well above its respective IgG Rabbit control antibody which is interpreted as a measure of assay background. In contrast, the 5-methylcytosine signal is low and frequently cannot be distinguished from its IgG Mouse control using endpoint PCR.

To obtain a quantitative difference between IDH wild-type and IDH mutant samples, qPCR was performed. Amplification of the 12S rRNA region of the mitochondrial genome (Figure 3-4), indicates that the introduction of the IDH1 R132H mutation results in a dramatic decrease in the level of 5hmC residues, about 4.5 fold relative to the levels of 5hmC in the IDH1 wild-type transfected sample. A slight increase in the abundance of 5mC residues relative to that found in the IDH1 wild-type sample is found in the mitochondrial genome when the IDH1 R132H mutation is expressed in this HCT116 cell line. However, the signal obtained from the 5-methylcytosine specific antibody is low, suggesting a low level of 5-methylcytosine residues in the mitochondrial genome. Background signal obtained with the mouse IgG control antibody is often as high as the 5mC antibody signal. Previous studies in the lab have shown substantial lot-to-lot variation in the background signal with mouse IgG. Multiple IgG mouse control antibodies will be tested in future experiments to more accurately determine the level of 5-methylcytosine modification found in the mitochondrial genome. However, since the level of 5-hydroxymethylcytosine found in these samples is well above baseline IgG control levels, it is reasonable to conclude that there is a preponderance of 5hmC modified DNA in the mitochondrial genome although the function of this modification in the mitochondria remains to be determined. When analyzing the level of 5hmC modified DNA in the 16S rRNA region after the incorporation of the IDH1 mutation, a similar trend is found as that seen in the 12S rRNA region (Figure 3-5). Once again, a 4.5 fold decrease in 5hmC signal is observed when mutant IDH1 is expressed compared to the IDH1 wild-type sample, indicating a substantial reduction in the amount of 5hmC in the 16S rRNA region of the mitochondrial genome. When analyzing the 5mC content present in the 16S rRNA region, we observed that the levels of 5mC increase in IDH1 mutated HCT-116 cells compared to the wild-type sample. However, once again, the

abundance of 5mC residues found in the 16S rRNA region of the mitochondrial DNA does not rise above the baseline IgG mouse control levels indicating a problem with this lot of IgG control antibody as previously mentioned.

Results obtained with transfection of the IDH2 R172K mutation, were somewhat different to those seen with the IDH1 R132H mutation. In IDH2 mutant transfected HCT116 cells, the levels of both 5mC and 5hmC are reduced when compared to their IDH2 wild-type counterparts. These results were similar in both the 12S (Figure 3-4) and 16S (Figure 3-5) regions of the mitochondrial genome. In the 12S rRNA region, there is a clear 4-fold drop in the levels of both 5hmC and 5mC. Examination of the 16S rRNA region indicates a more modest 2-fold decrease in 5hmC level when comparing IDH2 wild-type to IDH2 mutant while the amount of 5mC modified DNA present in this region is nearly 90% less in the IDH2 mutant sample compared to wild-type. Fortunately, the level of 5mC in both the ribosomal genes is above baseline (IgG control) in the IDH2 wild-type transfected cells however, the level falls below baseline with the introduction of the IDH2 mutation. The levels of 5mC and 5hmC were measured in the samples that were co-transfected with the D2 isoform of 2-HGDH as well since the (D)-isoform of 2-hydroxyglutarate is solely produced by these IDH mutations. With the introduction of this enzyme, the levels of 5hmC and 5mC begin to rise towards levels similar to those found in the IDH2 wild-type sample. In the 12S rRNA gene region, 5hmC and 5mC levels rise by 50% and 150% respectively compared to the IDH2 mutated samples. Since 2-HGDH functions to convert 2-HG to α-ketoglutarate, the effect of this enzyme was expected to reverse the decrease in 5hmC levels caused by mutant IDH through relief of competitive inhibition of the TET enzymes by 2-HG. The increase in 5mC levels in this co-transfected sample was opposite to that expected and likely reflects the very high IgG control in the mutant IDH samples

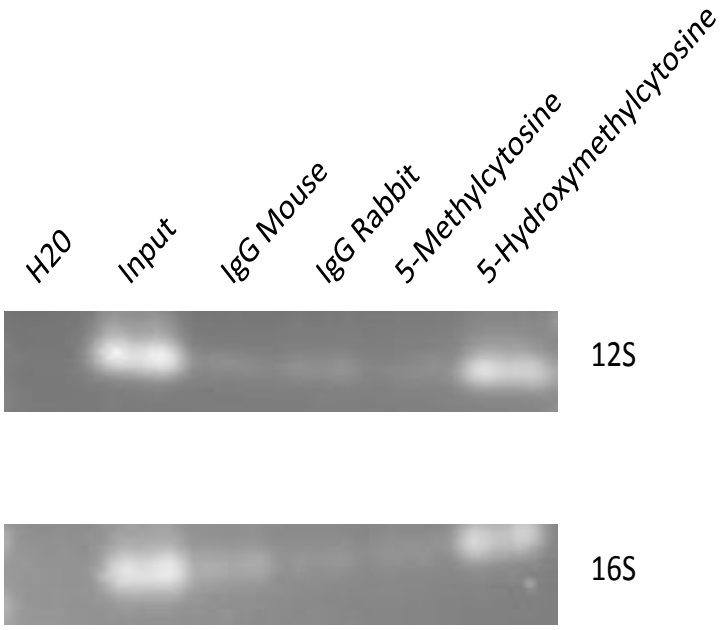

Figure 3-3: Example of endpoint PCR of HCT116 cells after transient transfection of IDH1 wild-type plasmid. DNA that was not used for MeDIP protocol was used for Input control. Both the 12S and 16S regions of the mitochondrial genome were analyzed using specific primers. PCR produce (200bp) was resolved on a 1% agarose gel. IgG Mouse compares to 5-methylcytosine while IgG Rabbit is compared to the signal produced by the 5-hydroxymethylcytosine antibody.

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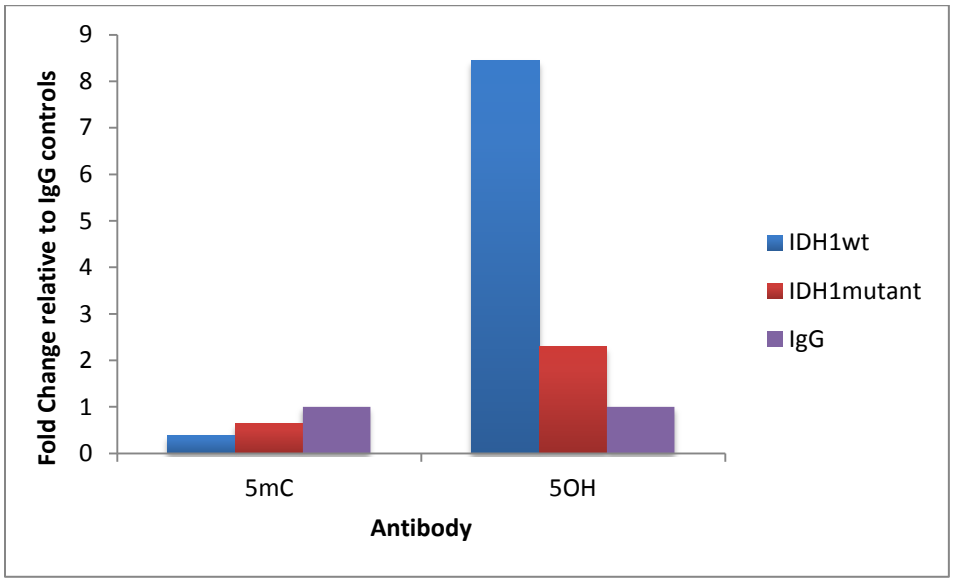


B

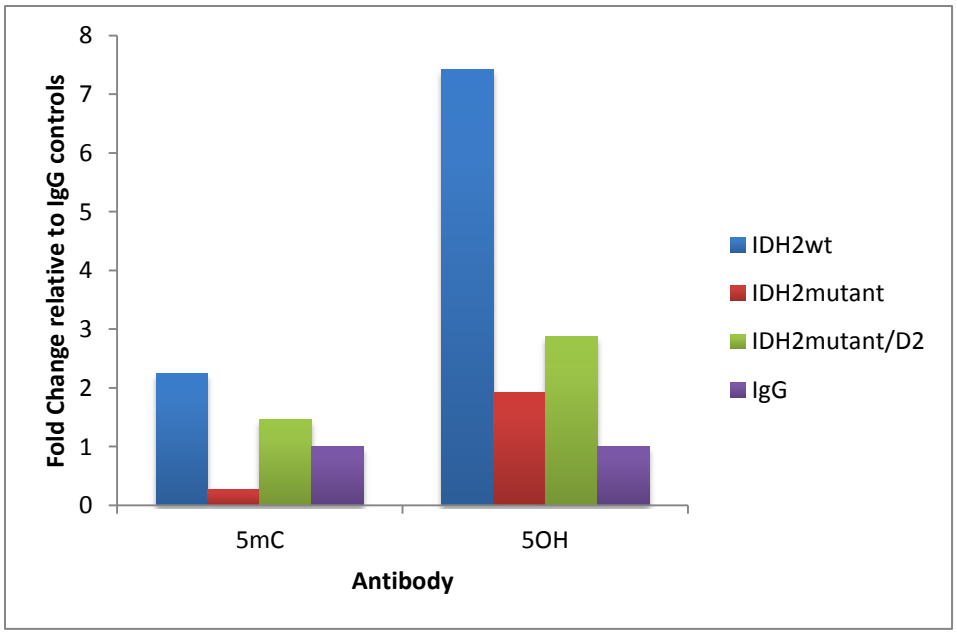
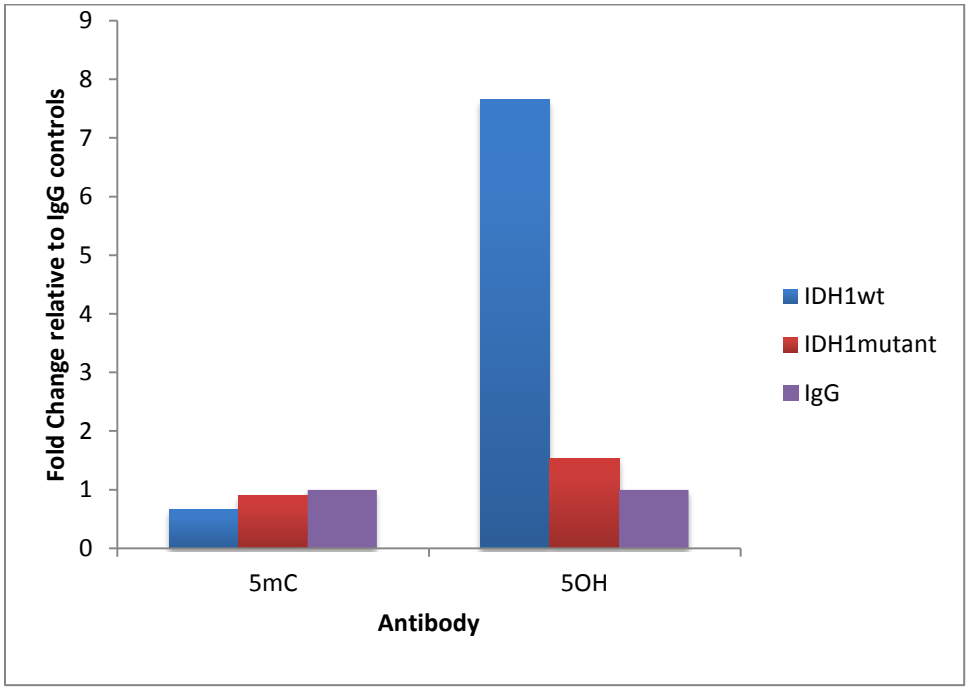


Figure 3-4: Expression of mutant IDH causes a reduction in the level of 5hmC in the 12S gene of the mitochondrial genome. Shown is a graphical representation of the effects of IDH enzymes on the abundance of 5-methylcytosine and 5-hydroxymethylcytosine in the 12S rRNA region of the mitochondrial genome. Samples were normalized to their respective IgG controls. Both IDH1 (A) and IDH2 (B) are displayed.

A



B

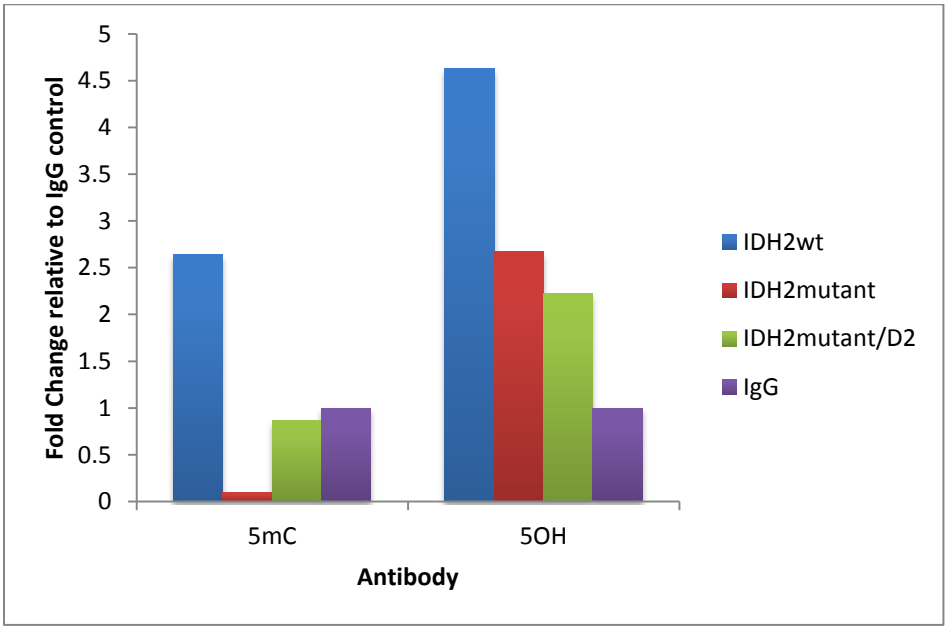


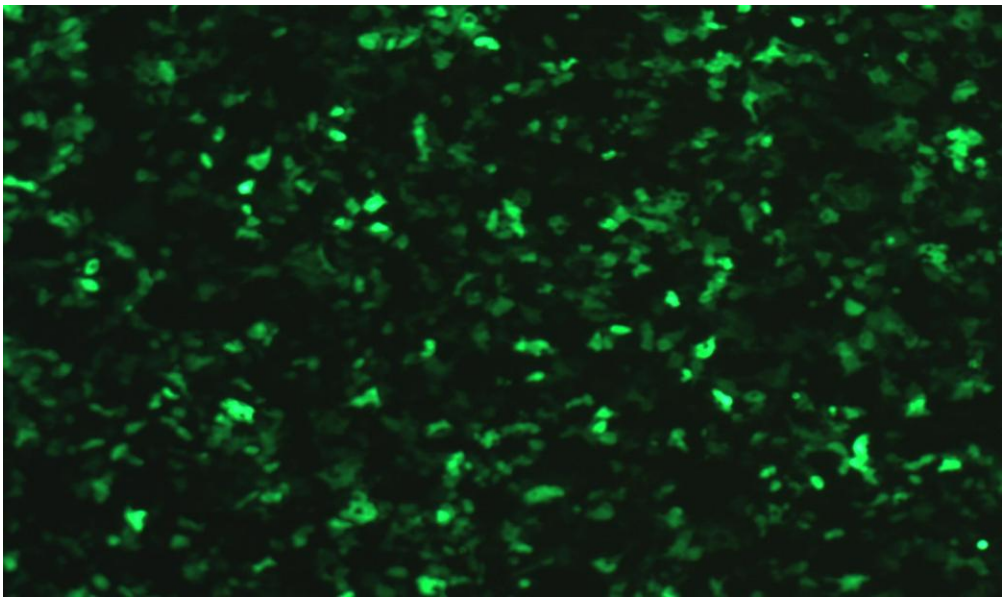
Figure 3-5: Expression of mutant IDH causes a reduction in the level of 5hmC in the 16S gene of the mitochondrial genome. Shown is a graphical representation of the effects of IDH enzymes on the abundance of 5-methylcytosine and 5-hydroxymethylcytosine in the 16S ribosomal RNA region of the mitochondrial genome. Samples were normalized to their respective IgG controls. Both IDH1 (A) and IDH2 (B) are displayed.

(Figures 3-4 and 3-5). However, a possible novel interaction between 2-HGDH and the methylation machinery of the mitochondria cannot be ruled out at this time. Further experiments will need to be performed to understand which of these two possibilities is at play. Interestingly, when analyzing the 16S rRNA gene cotransfection with IDH mutant and (D)-2HGDH, the level of 5mC increases by nearly 2-fold when compared to the level of 5mC found in the IDH2 mutated sample while the 5hmC level decreases by about 0.5 fold compared to IDH2 mutant levels. This result is different from thatfound in the nuclear genome and the 12S rRNA region, pointing to a possibly different mechanism of action for cytosine modification between these two ribosomal RNA regions of the mitochondrial genome.

The effect of IDH mutations on mitochondrial epigenetics using the HEK293 cell line

HEK293 cells were used to validate whether the effects of IDH mutations on mitochondrial epigenetics, observed using the HCT116 human cancer cell line, could be replicated in another cell line. The embryonic kidney cell line has been used by several other labs to uncover the role of IDH mutation on nuclear epigenetics and is much less mutated than the HCT-116 cell line. Using either wild-type or mutant IDH containing plasmids as well as co-transfections using the different 2HGDH isoforms (Table 1), transient transfection was performed once cells reached 70-80% confluency on their respective plates as previously described. Photomicrographs of the GFP plate were taken 24 hours and 48 hours after transfection to determine the efficiency of the transfection. As shown in Figure 3-6, about 50% of the cells fluoresce under the microscope after 24 hours post-transfection and about 90% of the cells fluoresce 48 hours after transfection. After protein concentrations were determined, immunoblotting was carried out as described using a VDAC loading control to assess equivalent loading of each of the samples onto the blot. Each of the IDH samples (samples 1-12)

A



B

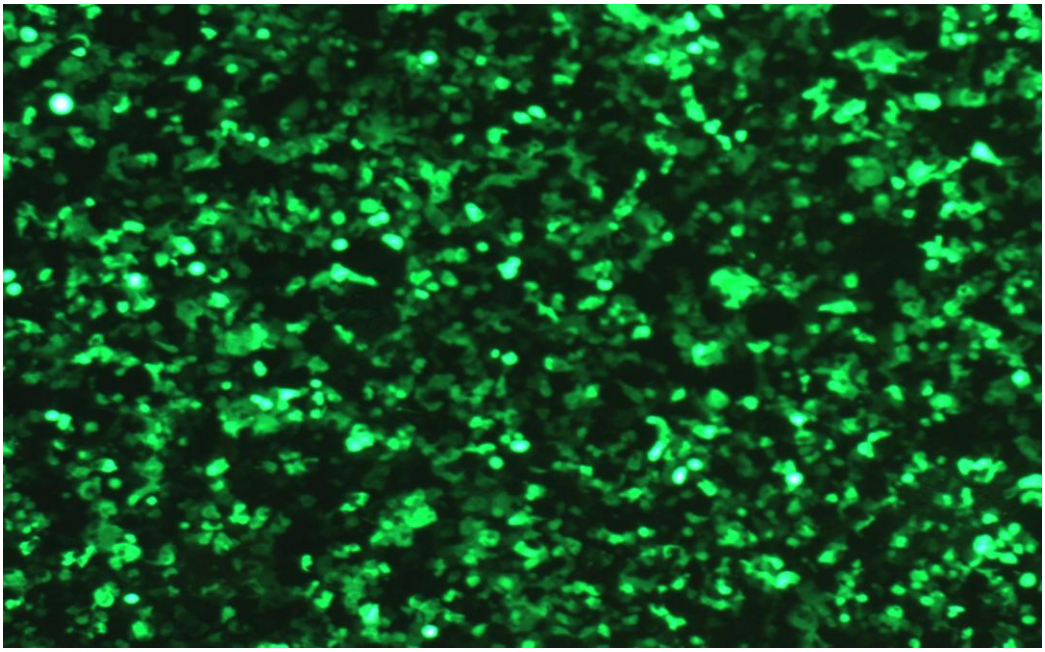


Figure 3-6: Determination of Transfection Efficiency of HEK293 cells. Microscopy image of GFP-expressing HEK293 cells 24 hours (A) and 48 hours (B) after initial transient transfection. These images were used as rough estimates of transfection efficiency.

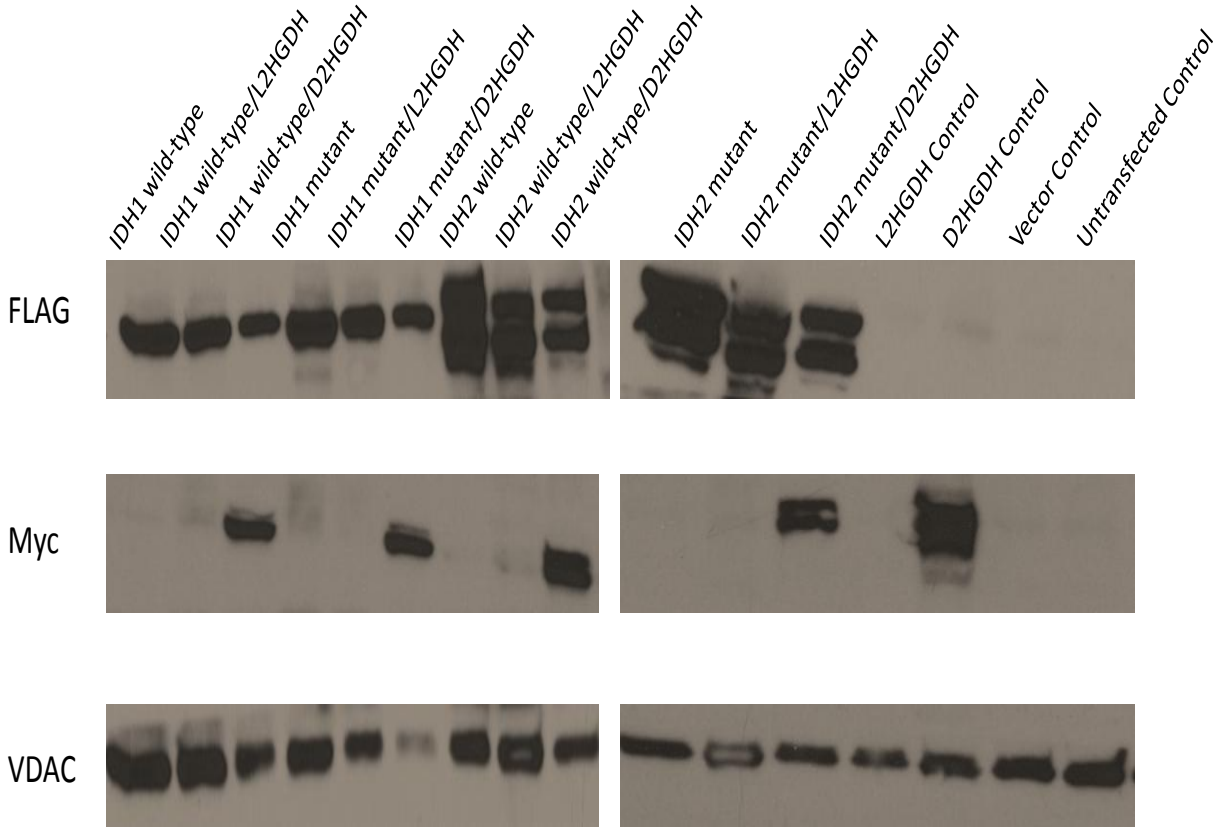


Figure 3-7: Immunoblots of transient transfection using HEK293 cell line. IDH proteins were analyzed using a FLAG-specific antibody while 2HGDH proteins were analyzed using a Myc antibody. VDAC was used as a loading control.

appropriately express the epitope-tagged protein on the plasmid that was transfected into the cells whether they were IDH1 wild-type or mutant or IDH2 wild-type or mutant (Figure 3-7). Interestingly, all IDH2 expressing plasmids displayed two bands at 46 and 50 kD. These bands represent the different subcellular locations of the protein as 39 amino acids are cleaved from the N-terminus upon entry into the mitochondria. The upper band represents the protein before mitochondrial localization as not all of the protein had entered this organelle before harvesting of the cells for protein extract while the lower band represents IDH2 protein that had entered the mitochondria. As before, the epitope tag encoded on the L2HGDH plasmid was not detectable. To detect this protein, an antibody directed against the protein itself may be necessary if available and alternative or N-terminal tags will be tested. The D2HGDH protein was expressed very well. No protein tagged with either FLAG or Myc was expressed in the blots of either the vector control or the GFP control used in this experiment indicating proper transfection of all samples. After determining that each sample expressed the appropriate proteins, the total cellular DNA was isolated. Pure DNA was sonicated to lengths between 200 to 500 base pairs and resolved on a 1% agarose gel to ensure that qPCR would detect modified residues only in the vicinity of the amplified fragment (Figure 3-8). For these experiments, the supernatant of both the IgG Mouse and IgG Rabbit samples were collected and used to determine input in order to control for variability in the amount of DNA used in each assay. The 5mC and 5hmC values would be measured as a percentage of input when compared to their IgG mouse and IgG rabbit controls respectively. Once the MeDIP was performed, endpoint PCR was used to validate the success of the IP (Figure 3-9). As can be seen, when amplifying the 12S and 16S rRNA regions of the mitochondrial genome, the IgG rabbit control gives a much fainter signal than its 5-hydroxymethylcytosine counterpart indicating an abundance of 5hmC in this sample.

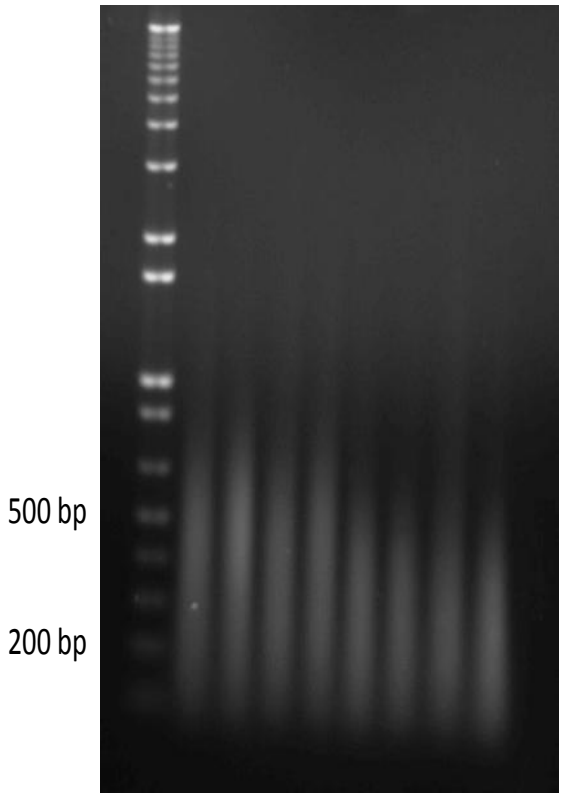


Figure 3-8: Sonication of purified DNA using HEK293 cells. Samples were sonicated between 200 to 500 bp to produce small enough DNA fragments useful for MeDIP protocol. Sonicated samples were resolved on a 1% agarose gel run for 70 minutes at 100 volts.

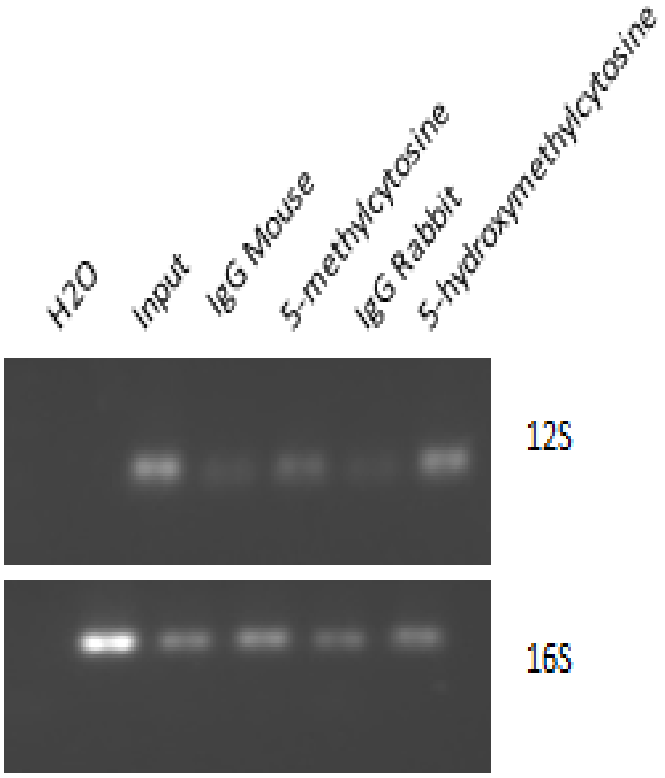


Figure 3-9: Endpoint PCR of HEK293 cells. Samples shown were transiently transfected with IDH1 mutant plasmid. DNA that was not used for MeDIP protocol was used for Input control. Both the 12S and 16S regions of the mitochondrial genome were analyzed using specific primers. The 200 bp amplicon was resolved on a 1% agarose gel.

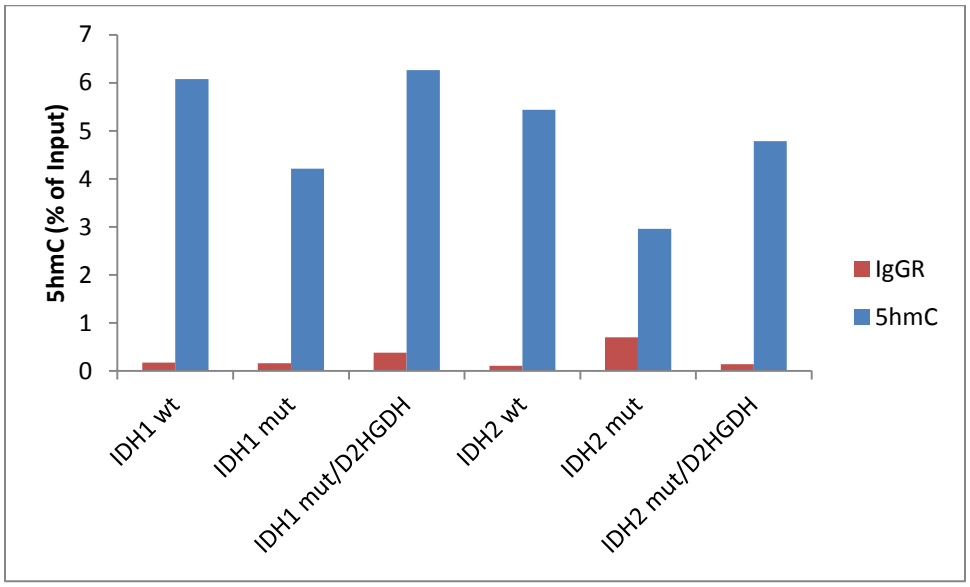
Unfortunately, the 5mC signal cannot be distinguished from its IgG mouse signal leading one to conclude that a difference in abundance of 5mC compared to background cannot be determined using the endpoint PCR method. It is possible that there is a much smaller amount of methylated cytosine residues in the mitochondrial genome. To better understand how IDH mutation affects the abundance of DNA modifications in the mitochondrial, qPCR was performed to obtain a quantitative measurement of whether and how the levels of 5hmC and 5mC areaffected following expression of IDH mutations.

As shown in Figure 3-10, in the 12S rRNA region of the mitochondrial genome, enrichment for 5hmC is substantially greater than the IgG rabbit control for each sample with the introduction of either IDH1 or IDH2 wild-type plasmids. This 5hmC signal is reduced by 50% or greater when IDH is mutated, most likely due to competitive inhibition of the TET enzymes by the 2-hydroxyglutarate produced by this enzyme. This effect is similar to that observed in HCT-116 experiments. The decrease in 5hmC content in HEK293 cells was partially rescued by co-transfection with the (D)-2-HGDH enzyme, which is expected to convert excess 2-HG to α-ketoglutarate, thereby partially relieving the inhibition placed on the TET enzymes by 2-HG. While restoration of 5hmC levels was observed by expression of the 2-HGDH enzyme, the levels did not reach those of the IDH wild-type samples. The amount of 2-HG produced by these mutant enzymes is substantial and may be too large for the 2-HGDH enzyme to convert back to α-ketoglutarate during the 48 hour time course of this experiment. Since the physiological concentration of the 2-HG oncometabolite is very low in cells (0.1nM), the kinetic parameters of this enzyme in the face of millimolar concentrations are not yet characterized. It is also important to keep in mind that the data presented here represent one of three biological replicates and the data for the other replicates are still being analyzed.

The 16S rRNA region, showed similar results to those shown in the 12S rRNA region. The wild-type IDH1 5hmC level is high compared to its IgG rabbit control (Figure 3-11), while transfection with plasmid expressing mutant IDH1 results in a 50% reduction in the level of 5hmC in comparison to the IDH wild-type level, indicating loss of 5hmC at the 16S rRNA region of the mitochondrial genome. Co-transfection with (D)-2-HGDH partially restores the level of 5hmC, likely due to the relief of the inhibition placed on the TET enzymes by 2-HG. Surprisingly, for IDH2, the mitochondrial isoform of this enzyme, the levels of 5hmC in the 16S region, are very similar between IDH2 mutant and wild-type IDH2-transfection. This trend is somewhat different from that observed in the HCT-116 cell line and in nuclear genomic DNA once again demonstrating the possibility that these different mitochondrial genes react independently of each other to stimuli. Again, it is also important to realize that this is one single biological repeat of three so the trend may be specific to this repeat and will need to be analyzed along with the other repeats to determine the biological effect of this mutated enzyme. Since the concentration of α-ketoglutarate in the mitochondria is much greater than in the cytoplasm, the conversion of this metabolite to 2-HG may not be as pronounced when IDH2 is mutated as compared to IDH1. Mass spectrometry will be used in future experiments to determine the level of 2-HG produced by each mutation.

The changes in levels of 5-methylcytosine in DNA samples from these transfected HEK cells were inverse to those seen in the levels of 5-hydroxymethylcytosine for IDH1 transfections. When the IDH1 wild-type plasmid was introduced into HEK293 cells the levels of 5mC were below the baseline levels determined by the IgG controls in both the 12s and 16s regions of mitochondrial DNA but the introduction of a IDH1 R132H mutation brought about a rise, above baseline, in the levels of methylation found in these ribosomal gene regions. This low signal

A. 5hmC



B. 5mC

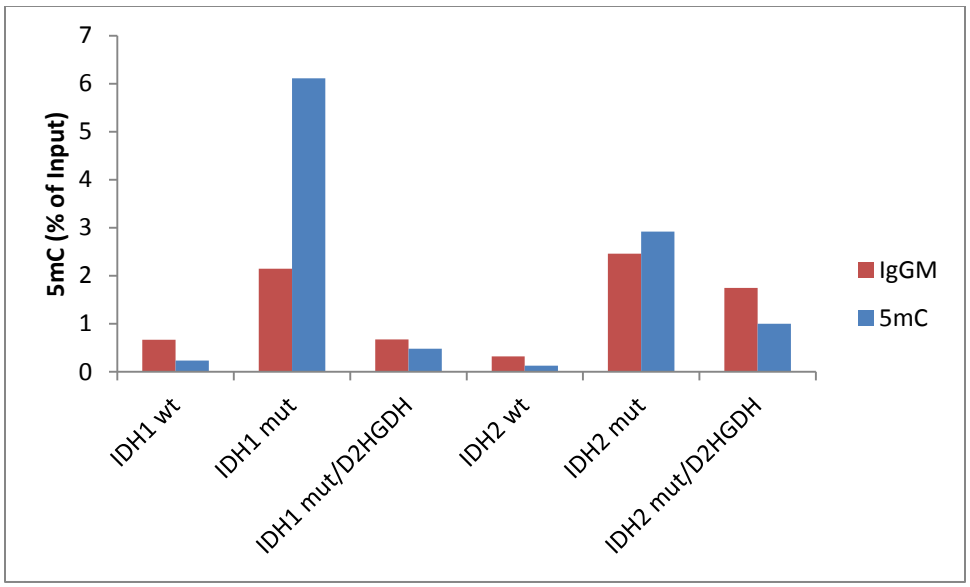
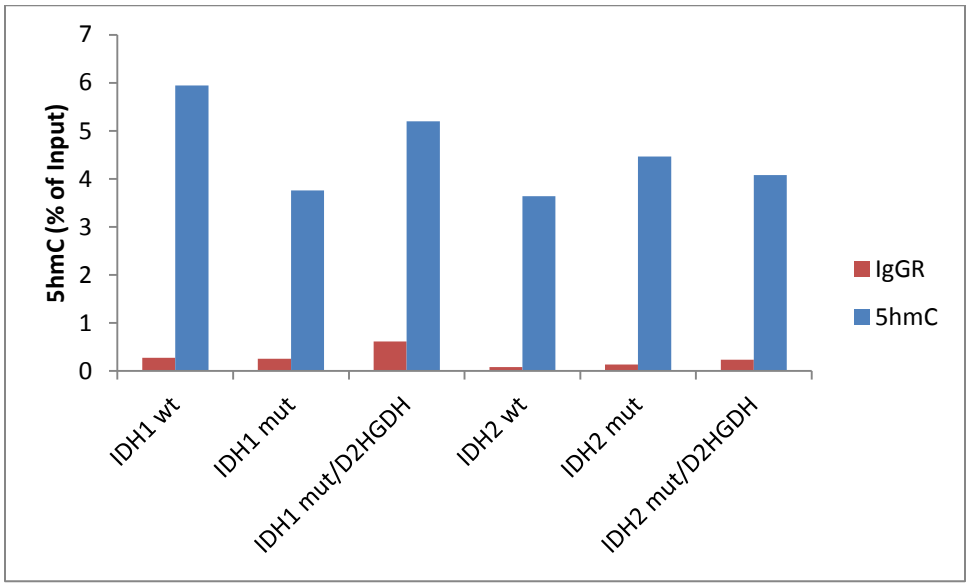


Figure 3-10: Analysis of epigenetic modification of the 12S rRNA gene after transfection of HEK293 cells with wt and mutant IDH. The abundance of 5-hydroxymethylcytosine (A) and 5-methylcytosine (B) residues found in the 12S ribosomal RNA region of human mitochondrial DNA after transfection of HEK293 cells expressed as % of input DNA.

A. 5hmC



B. 5mC

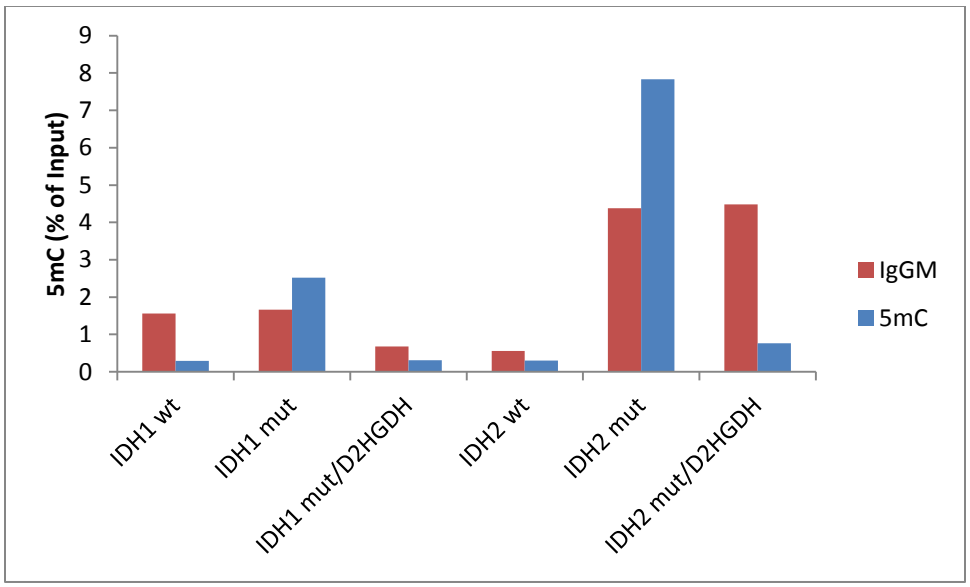


Figure 3-11: Analysis of epigenetic modification of the 16S rRNA gene after transfection of HEK293 cells with wt and mutant IDH. The abundance of 5-hydroxymethylcytosine (A) and 5-methylcytosine (B) residues found in the 16S ribosomal RNA region of human mitochondrial DNA after transfection of HEK293 cells, expressed as % of input DNA.

makes it difficult to determine the levels of 5mC in the mitochondrial genome when IDH is not mutated and thus these samples cannot be adequately compared to each other. It is also difficult to establish a trend since the levels of the IgG mouse control antibody differ drastically between each sample. This technical issue is currently being resolved in the lab.

A similar trend exists when IDH2 is mutated in that the percentage of methylated cytosine residues rises compared to the levels found in IDH2 wild-type samples. Also of interest is that when IDH2 mutant samples were co-transfected with (D)-2-HGDH, the level of 5mC decreased which may mean that this enzyme is working to convert excess 2-HG back to α-ketoglutarate thereby relieving the inhibition placed on the TET enzymes and allowing for more hydroxylation of 5-methylcytosine residues. This trend is similar in both the 12S and 16S rRNA gene regions. This experiment was repeated in three separate biological repeats using HEK293 cells however, as previously mentioned, only one repeat is shown in the figures above. This data is currently being analyzed further to determine if the trends above are consistent between each biological repeat. Also, since the IgG mouse control antibody provides different levels of enrichment between samples, it is not possible to accurately determine how IDH mutations affect the methylation of cytosine residues in the mitochondria, and we will test different mouse IgG samples to obtain material that consistently yields low background values..

The effect of Long-Term Expression of IDH mutants

To determine how IDH mutations affect the mitochondrial epigenome over time, cell lines stably expressing the IDH1 wild-type and mutant cDNAs, as well as those expressing IDH2 wild-type and mutant cDNAs, were created. This experiment was performed to determine if a similar hypermethylator phenotype as that produced in the nuclear genome develops in the

mitochondria and determine whether these mutant enzymes cause a change in mitochondrial gene expression or metabolic function of the mitochondria. This experiment was performed using HEK293 cells and cells were transfected and subjected to neomycin selection (1mg/mL) to eliminate cells that did not integrate the IDH plasmids into their genomes. As this DNA is integrated randomly into the genome and might be present in multiple copies, immunoblotting was performed to determine the level of expression of the transgene in each clone. Since these integration events are random and can occur multiple times in the genome, many clones were isolated for each plasmid and analyzed for protein expression using the FLAG antibody. To control for these multiple integration events, master cultures were also created, analyzed for protein expression and frozen for later use to determine the overall average effect of these mutant IDH proteins. Once confirmed, three clones of each IDH-expressing sample were analyzed to determine if the level of this enzyme being expressed was physiologically relevant to the levels found in normally untransfected cells. This was done to ensure that changes in the levels of 5hmC and 5mC were relevant to those found in human tumor samples. The endogenous levels of IDH were determined using an antibody specific for IDH2 that was able to recognize both the IDH1 and IDH2 isoforms present in the cell. These stable clones were compared to untransfected control HEK293 cells to determine the degree of overexpression of each in the stable cell culture. VDAC was used as a loading control (Figure 3-12) as well as a Cyclophilin A antibody which recognizes a ubiquitously expressed nuclear receptor protein. The Cyclophilin A antibody was used for the IDH2 expressing clones (Figure 3-13) since we observed fluctuating levels of VDAC signal in these experiments, possibly related to effects on mitochondrial integrity. The clones that were chosen (Figure 3-12 & Figure 3-13) expressed their FLAG-tagged IDH plasmids. All clones expressed either equivalent or greater amounts of the IDH protein,

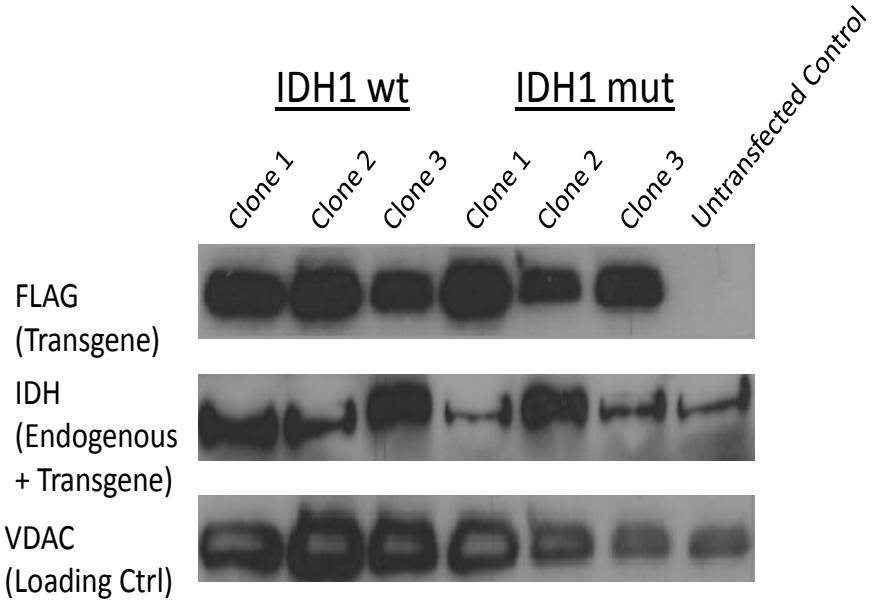


Figure 3-12: IDH expression levels in IDH1 mutant and wild-type stable clones. Three separate wild-type clones as well as three separate IDH1 mutant expressing clones were analyzed to determine level of transgene expression. An untransfected control was used to determine levels of endogenous IDH expressed in HEK293 cells. VDAC was used as a loading control to assess equal loading of each sample.

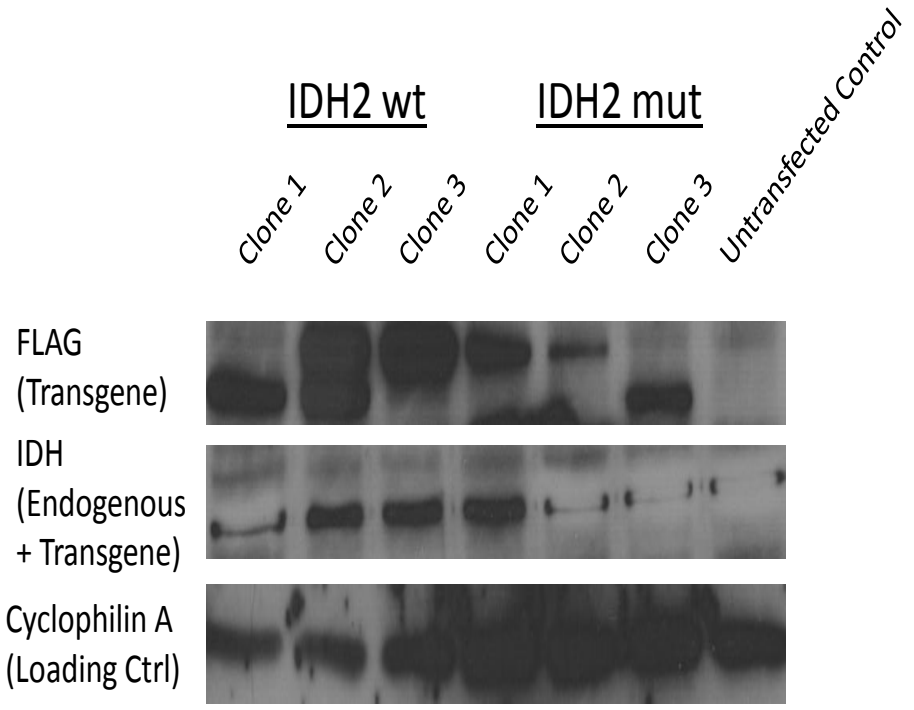


Figure 3-13: IDH expression levels in IDH2 mutant and wild-type stable clones. Three separate wild-type and mutant expressing clones were analyzed to determine level of transgene expression. An untransfected control was used to determine levels of endogenous IDH expressed in HEK293 cells. Cyclophilin A was used as a loading control to assess equal loading of each sample.

indicating a level of expression equivalent or greater to the endogenous levels of the IDH protein in HEK293 cells. In regards to the IDH2 samples, different band sizes were observed indicating the degree of transport into the mitochondria since the mitochondrial leader peptide is cleaved after the protein enters the mitochondria. As the protein is being adequately expressed in these clones, two clones (the second and third clone also) were kept. The second and third clones of each sample were kept and cells will be grown over time to determine how the levels of 5-methylcytosine and 5-hydroxymethylcytosine change over multiple passages in the mitochondrial genome by utilizing the MeDIP technique.

Effect of Octyl-2-hydroxyglutarate on the 5-hydroxymethylcytosine content of the mitochondrial genome

As it is known that the production of 2-HG has a pronounced inhibitory effect on the TET dioxygenase family and causes a change in the epigenome of mammalian DNA, cells were treated with a cell-permeable analog of this oncometabolite, octyl-(D)-2-hydroxyglutarate, to determine how it affects the 5-methylcytosine content of the mitochondrial genome, specifically the 12S and 16S ribosomal regions. Mutant IDH also oxidizes NADPH to $NADP^+$ thereby decreasing the stores of NADPH available in the cell. This may be detrimental to the cell as NADPH is necessary for many cellular processes such as macromolecular biosynthesis and antioxidant properties. In all tumor samples analyzed to date, the IDH mutations solely produce the D-isoform of 2-HG so this experiment should replicate what is found *in vivo*. This analog is able to penetrate both the cell and mitochondrial membranes and would be expected to affect processes in all compartments. Also, using this molecule, we bypass any adverse effects of an imbalanced NADPH/NADP+ ratio on the mitochondria with respect to oxidative phosphorylation and energy production. HEK293 cells were treated with either 1μM or 10μM

octyl-(D)-2-HG and DNA was harvested for MeDIP analysis. We included a no drug and DMSO control, since the octyl-2-HG is dissolved in DMSO. After MeDIP, endpoint PCR was performed to obtain a visual representation of how the different levels of 5mC and 5hmC appear in the mitochondria using primer sets for the 12S and 16S rRNA regions of the genome (Figure 3-14). Results of endpoint PCR were similar to past examples in that the 5hmC signal was much greater compared to its IgG rabbit control while the 5mC signal remained difficult to distinguish from the signal generated by the IgG mouse control.

When analyzed using qPCR, the level of 5-hydroxymethylcytosine decreased by about 50% in the 12S rRNA region and almost 90% in the 16S rRNA region (Figure 3-15) in samples treated with octyl-2-HG as compared to the untreated controls. The DMSO control also had reduced 5hmC content in the mitochondrial genome indicating an effect of this molecule on mitochondrial epigenetics. Interestingly, the level of 5hmC remained constant even after a ten-fold increase in octyl-(D)-2-HG concentration suggesting that TET enzymes can be inhibited fairly easily by this metabolite at low concentrations and that the concentration of this molecule is tightly regulated in cells. The decrease in 5hmC produced by this drug is similar to that produced by the mutant IDH proteins implying that the amount of 2-hydroxyglutarate necessary for inhibition of TET enzymes that locate to the mitochondria is between 1-10μM and can produce an effect on mitochondrial epigenetics fairly quickly.

The effect of the cell permeable analog of 2-hydroxyglutarate on 5mC levels in HEK 293 cells was again difficult to assess, due to the high and very variable background signal seen with the mouse IgC antibody control, (Figure 3-16) in both the 12S and 16S regions of this genome. Also of note, is that the DMSO vector control, which has a negative effect on 5hmC content in the mitochondria, may also have an effect on 5mC levels in the organelle although the

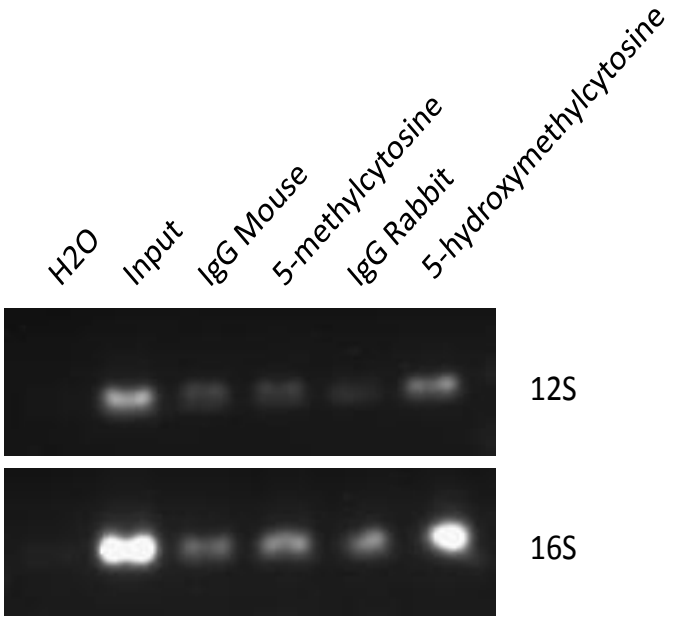
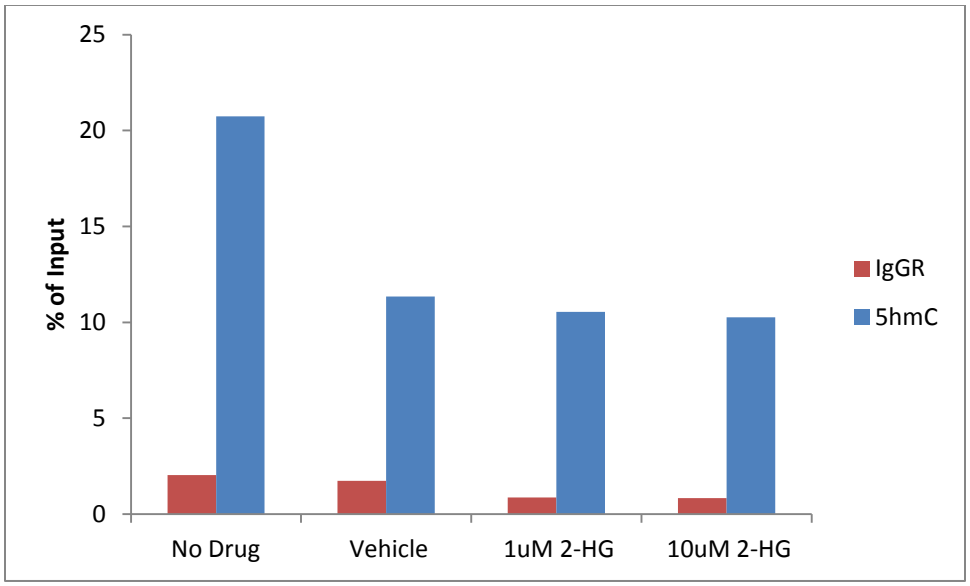


Figure 3-14: Visual representation of 12S and 16S rRNA regions after treatment with cell permeable 2-hydroxyglutarate. Example of endpoint PCR of immunoprecipitated DNA from HEK293 cells after treatment with 1μM octyl-(D)-2-hydroxyglutarate. Both the 12S and 16S rRNA regions of the mitochondrial genome were analyzed using specific primers. The 200bp amplicon was resolved on a 1% agarose gel.

A.



B.

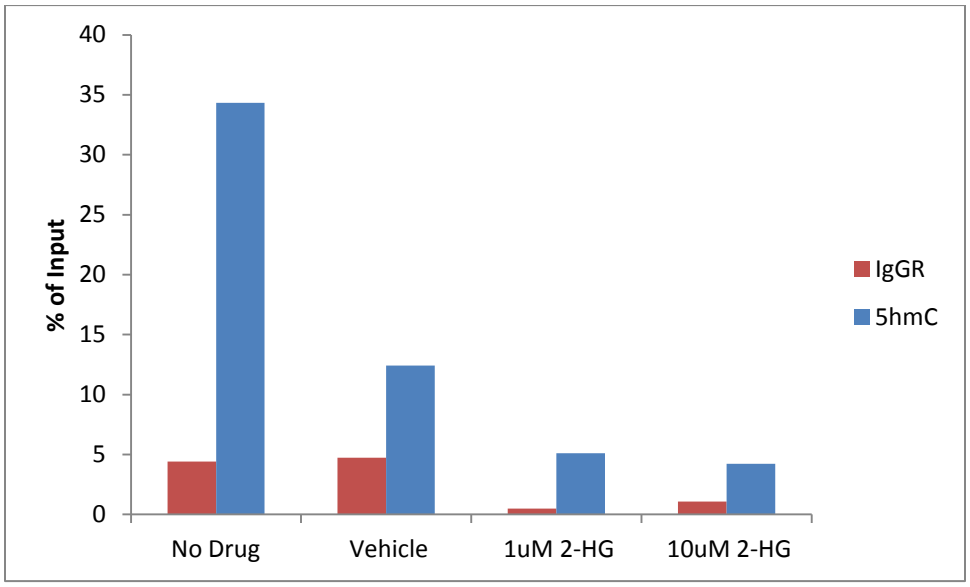
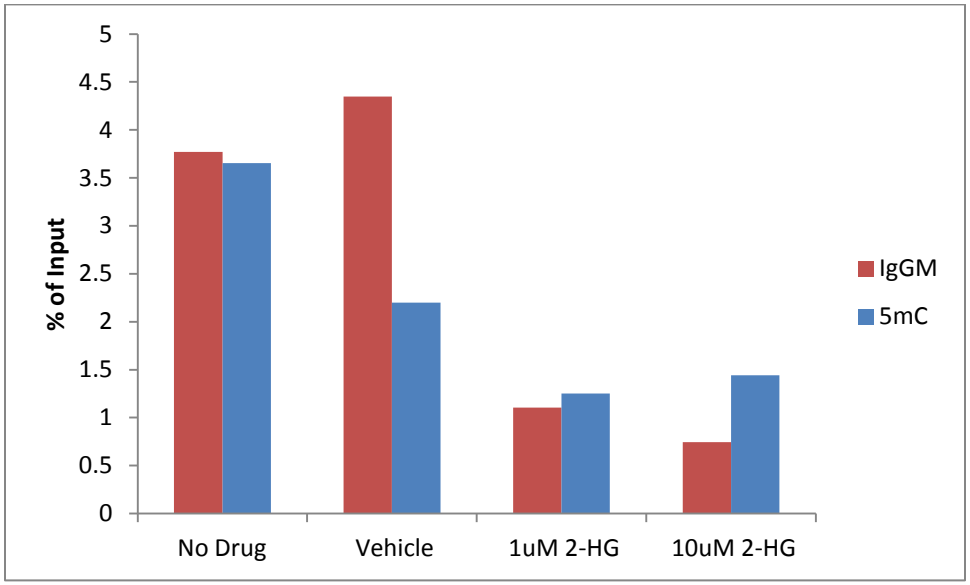


Figure 3-15: Treatment of HEK293 cells with cell permeable 2-hydroxyglutarate results in reduced 5hmC content in the mitochondrial genome. 12S (A) and 16S (B) rRNA gene regions were analyzed after treatment with 1μM and 10μM concentrations of octyl-(D)-2-HG.

A



B

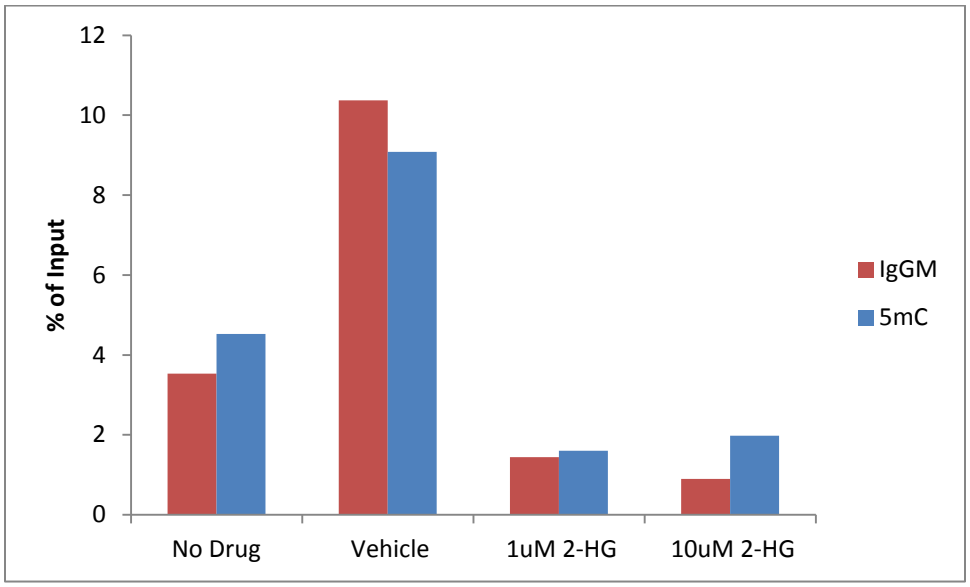


Figure 3-16: Treatment of HEK293 cells with cell permeable 2-hydroxyglutarate results in indeterminate changes in 5mC content in the mitochondrial genome. 12S (A) and 16S (B) rRNA gene regions were analyzedafter treatment with 1µM and 10µM concentrations of octyl-(D)-2-HG.

signal produced is below the IgG baseline value making any comparison impossible. This experiment provides clear evidence that 2-HG causes a competitive inhibition of the enzymes necessary for hydroxymethylation of the mitochondrial genome. However, an effect of the oncometabolite on cytosine methylation of the mitochondrial genome is still an open question.

Use of an IDH1 Mutation Inhibitor to Rescue 5-hydroxymethylcytosine levels

To more fully understand the effect of IDH mutations in tumorigenesis and to determine if the effects can be reversed and used as a clinical therapeutic, a company headquartered in Cambridge, Massachusetts (Agios) developed a molecule that can specifically inhibit the IDH1 R132H mutation and restore cellular differentiation potential and nuclear epigenetic marks while not affecting IDH1 wild-type tumors (62). As the molecule developed by Agios to inhibit IDH2 was created against the IDH2 R140Q mutation, our studies focused on the IDH1 mutant inhibitor which is specific for the R132H mutation (80). If the new activity of mutant IDH, which produces 2-HG, is responsible for a reduction in 5hmC in the mtDNA, we would expect that specific inhibition of this activity would restore levels of 5hmC in the mitochondrial DNA. Accordingly, we tested whether a similar result could be achieved in mitochondrial DNA and restore the level of 5hmC in this genome thereby relieving inhibition of the TET enzymes by 2-HG. AGI-5198 (5μM) was incubated with HEK293 cells that stably express the IDH1 R132H mutation. Drug treated cells were harvested at different time points and pelleted for DNA purification to determine the effects of this drug on the mitochondrial epigenetics of the cell. An untreated and DMSO treated sample were used as controls for this experiment. Afterwards, MeDIP was performed to harvest all of the DNA molecules that contain either 5-methylcytosine or 5-hydroxymethylcytosine residues. After MeDIP, endpoint PCR using primer sets for the 12S and 16S rRNA regions of the mitochondrial genome was performed to determine the success rate

of the IP prior to proceeding to qPCR. An example of the end-point PCR is shown in Figure 3-17. Once again, the signal produced by the 5hmC sample was much more apparent than its IgG rabbit control while the level of 5mC in the mitochondrial ribosome genes remained diminished and close to the level of the IgG mouse control. It is apparent that levels of methylation in the two ribosomal regions of the DNA are kept at a minimal level while hydroxymethylation remains high in these areas.

qPCR was employed as before to determine if the quantity of 5hmC residues in the mitochondrial genome increases after treating cells with AGI-5198. Treatment with this drug does in fact result in an increase in 5hmC content in the 12S and 16S region of mtDNA (Figure 3-18). In the 16S rRNA region, there is a time-dependent response to the IDH1 mutant inhibitor as the level of 5hmC rises above the level of the no-drug control when exposed to this drug for short (48 hours) and long (144 hours) periods of time. In the 12S rRNA region, a positive trend exists between 5hmC levels and time exposed to AGI-5198. However, it is interesting to note that when evaluating the 12S region, the percentage of the 5hmC modification is greater than either of the treated samples indicating a possible technical problem with this assay. Cells used for the no-drug control will need to be analyzed to determine if the IDH1 mutation is expressed appropriately.

The level of 5-methylcytosine content was also measured in these AGI-5198 treated cells. As seen in Figure 3-19, this drug has an inverse effect on the level of 5mC in the mitochondrial genome. When treated with AGI-5198 over time, the level of 5mC in the 12S rRNA region decreases to that of the IgG control. This pattern is similar to that seen in the IDH1 wild-type transfected cell samples, indicating that the levels of 5-methylcytosine in the 12S region are kept

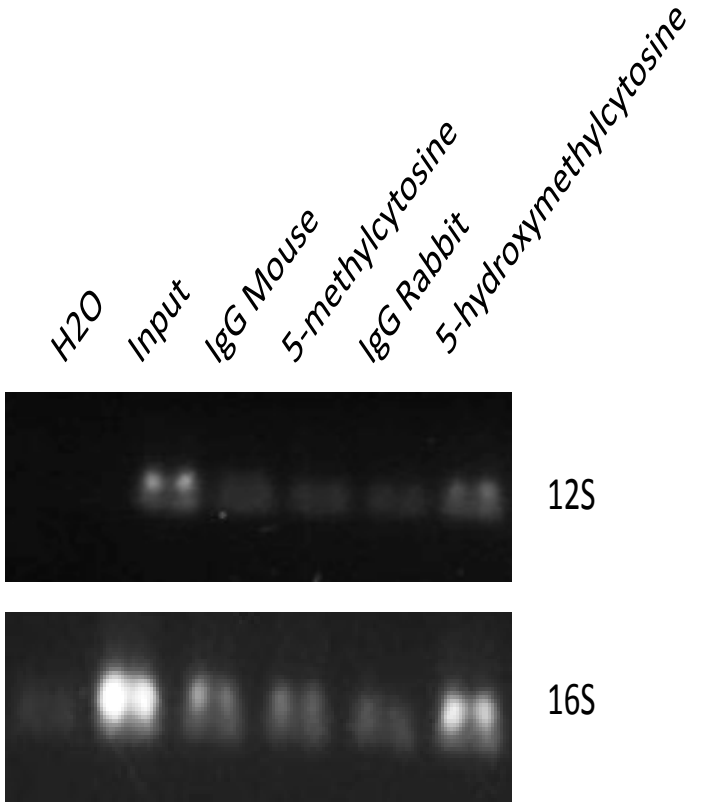
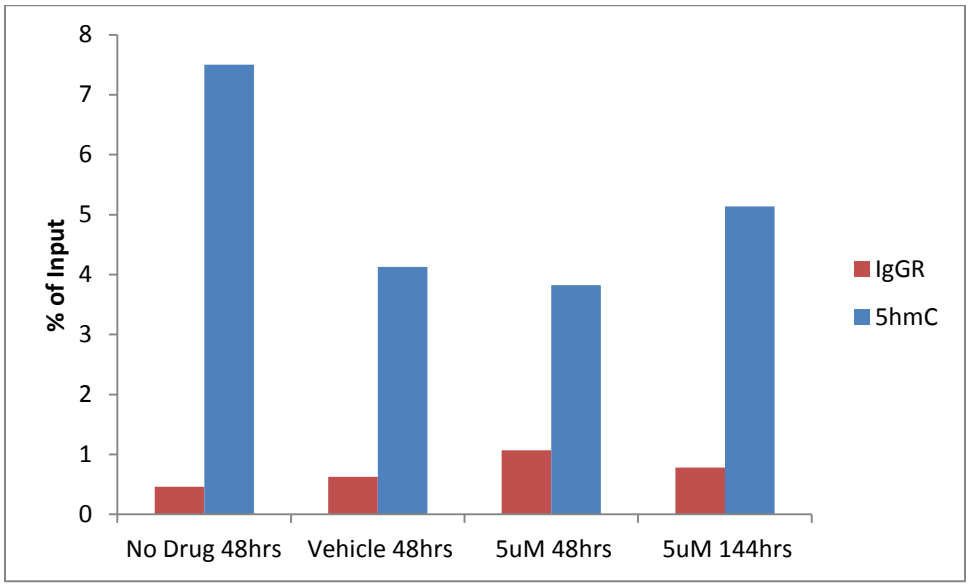


Figure 3-17: Visual representation of 12S and 16S rRNA regions after treatment with IDH1 mutant inhibitor. Example of endpoint PCR of HEK293 cells used in experiment to determine how AGI-5198 affects mitochondrial epigenetics. Both the 12S and 16S rRNA regions of the mitochondrial genome were analyzed using specific primers. The 200bp amplicon was resolved on a 1% agarose gel.

A



B

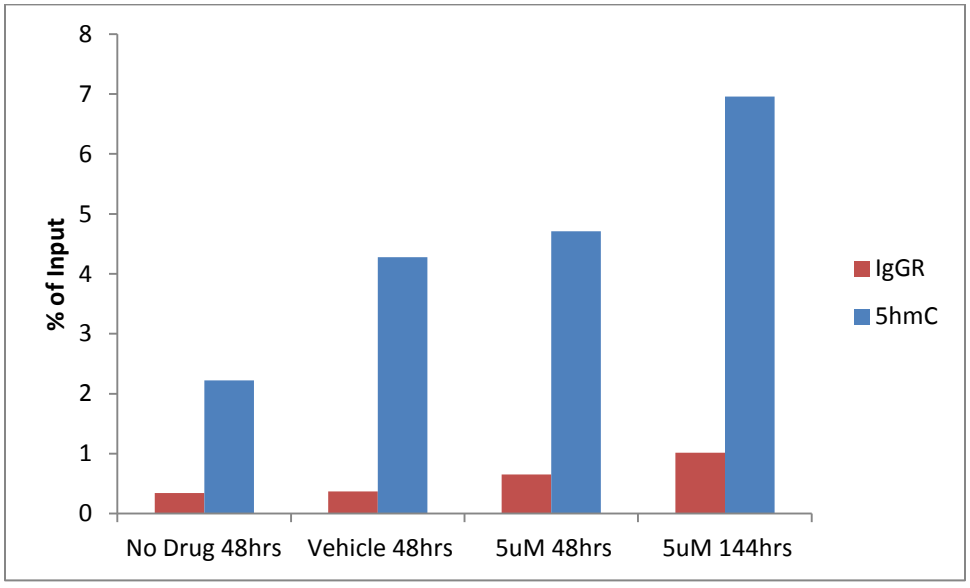
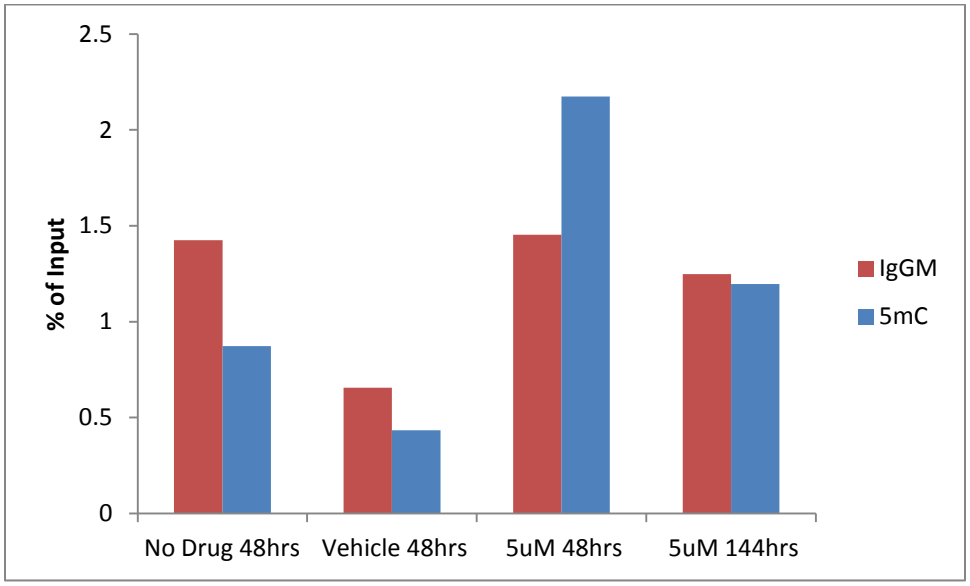


Figure 3-18: Evaluation of levels of 5hmC after exposure to a specific inhibitor of mutant IDH1. IDH1 mutant expressing cells were treated with AGI-5198 for 48 or 144 hours to determine if levels of 5hmC could be restored in the mitochondrial 12S (A) and 16S (B) rRNA regions.

A



B

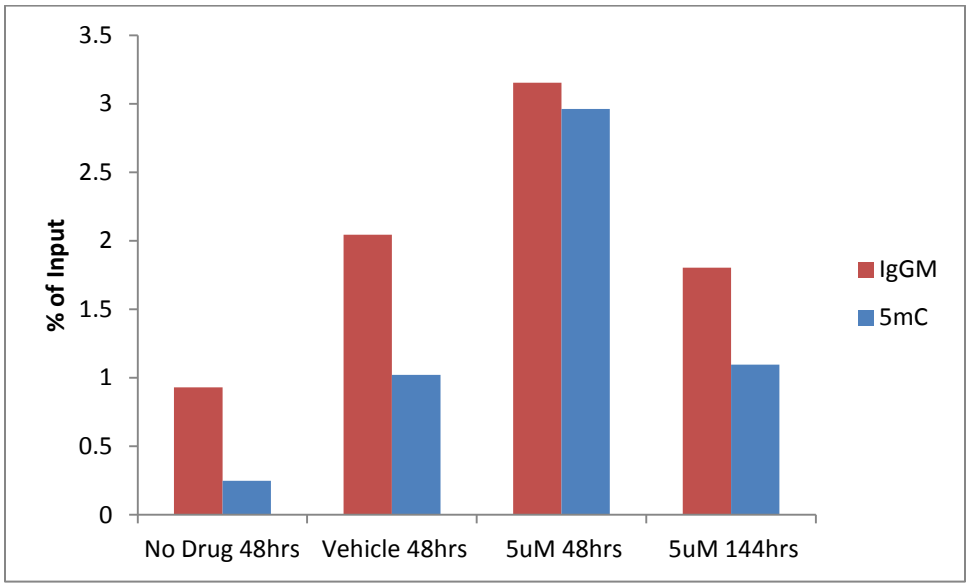


Figure 3-19: Evaluation of levels of 5mC after exposure to a specific inhibitor of mutant IDH1. IDH1 mutant expressing cells were treated with AGI-5198 for 48 or 144 hours to determine if levels of 5mC could be reduced in the mitochondrial rRNA regions. Both the 12S (A) and 16S (B) regions were analyzed.

at a minimal level. In the 16S rRNA region, all the treated samples are below their respective IgG controls making it difficult to determine if a correlation exists between IDH1 mutant inhibition and the level of 5mC in the mitochondria. As the level of 5mC is so low in the no-drug treated controls, it is possible that the cells used did not accurately express the IDH1 R132H mutation and will need to be analyzed to determine expression of this enzyme in this cell line. These results indicate that with the addition of the IDH1 mutant inhibitor AGI-5198 to cells expressing this aberrant enzyme, the level of 5hmC can begin to be restored to IDH1 wild-type levels with a simultaneous decrease in the level of 5mC over time. This compound inhibits the oncogenic function of mutant IDH1in producing 2-HG from α-ketoglutarate, thereby relieving inhibition of TET enzymes and allowing for adequate hydroxylation of 5-methylcytosine residues in the mitochondrial DNA.

Chapter 4: Discussion

Aberrant Metabolism in Cancer: Understanding the Warburg Effect

In the 1950s Dr. Otto Warburg published his seminal paper on the theory of aerobic glycolysis in cancer, which stated that cancer cells preferentially produce ATP via lactate production in the glycolytic pathway instead of using the oxidative phosphorylation pathway typically used by differentiated cells (81). Since then, much work has been done to try to understand this hypothesis.

In normal cells, ATP is commonly produced via the electron transport chain (ETC) found exclusively in the inner membrane of the mitochondria, the "powerhouse" of the cell (Figure 4-1). This process utilizes NADH, NADPH and $FADH_2$, known electron acceptors produced via the tricarboxylic acid cycle, to carry electrons to the different complexes of the ETC. These electrons are produced via dehydrogenase reactions in the mitochondria in the form of a hydride ion (H^-). The dehydrogenase reaction receives two hydrogen atoms from its substrate, the hydride ion just mentioned and a hydrogen ion (H^+) which is fluxed out of the mitochondrial inner membrane. The electrons produced are carried between the electron acceptors of the five complexes of the oxidative phosphorylation chain where they end up combining with O_2 to create a water molecule. Three of the five complexes (Complex I, III, and IV) subsequently produce a hydrogen ion gradient as electrons are passed through the ETC. This proton gradient is utilized by the fifth complex in the electron transport chain, ATP Synthase to produce ATP from ADP and inorganic phosphate (P_i). This process is typically regulated by the ADP/ATP ratio available as a measure of energy demands for the cell.

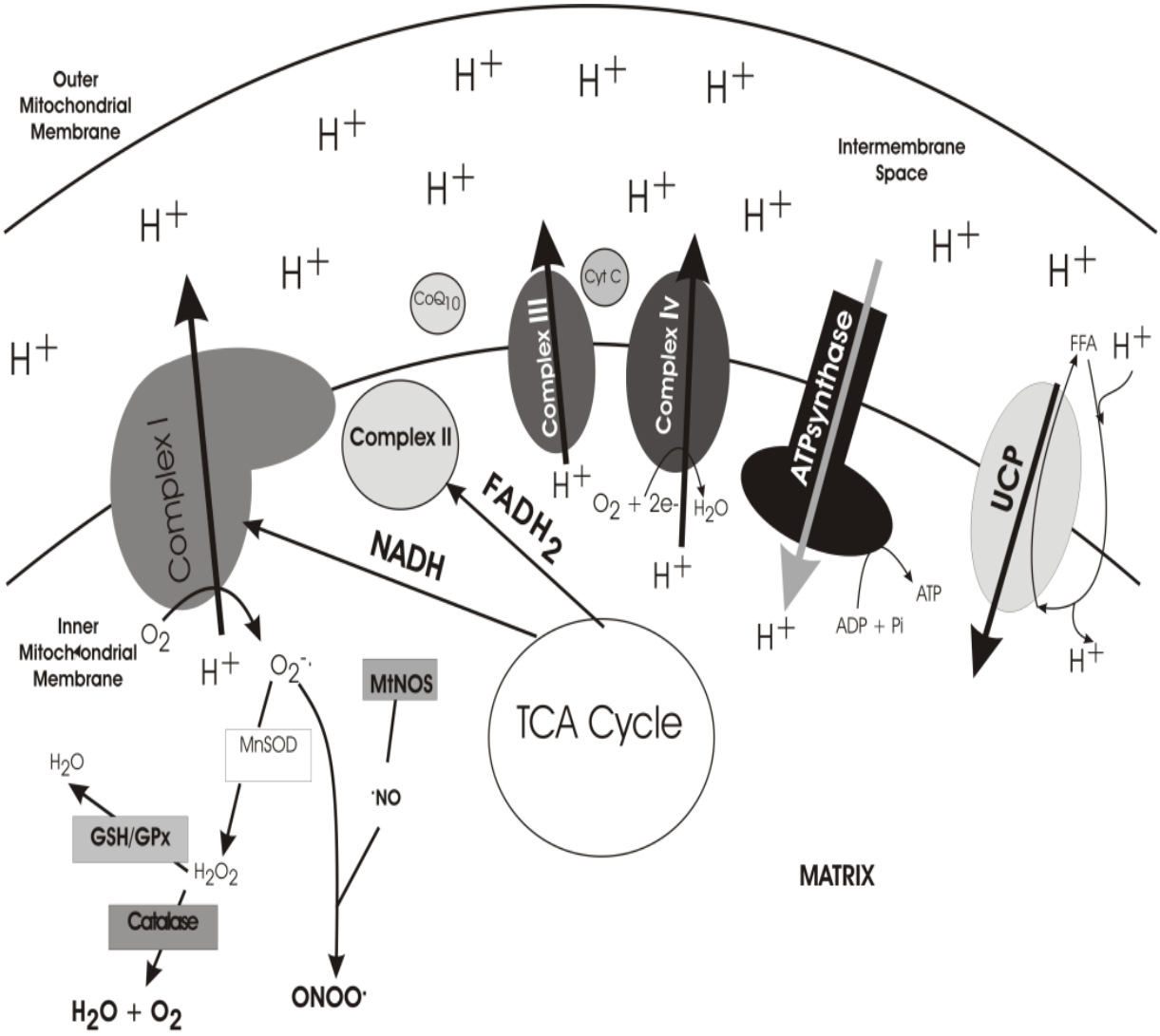


Figure 4-1: Diagram of oxidative phosphorylation as it occurs in normally dividing cells within the inner membrane of the mitochondria. NADH is produced via the TCA cycle and shuttled to Complex I where electrons are moved to differing complexes to create a proton gradient. Proton gradient is utilized in the generation of ATP by Complex V (ATP Synthase). Figure adapted from Readnower, 2011.

In cancer, cells use a different pathway to achieve rapid ATP production. This pathway, termed aerobic glycolysis, utilizes the glycolytic pathway to convert glucose into lactate. In normal cells, this pathway operates in a hypoxic environment when energy demands are high such as in strenuous exercise. Although glycolysis is much faster at producing ATP, less ATP is produced (36). Cancer cells typically utilize this pathway for faster energy production, even in the presence of oxygen, to maximize tumor growth. While it is debated as to why this shift in ATP production occurs, the rapid uptake of glucose by cancer cells has been employed as a clinical tool in tumor screening and detection. [^{18}F]fluorodeoxyglucose positron emission tomography imaging, or FDG-PET, has been used to detect areas of high glucose uptake in the body by using radioactively labeled glucose analogs and has proved highly effective in detecting and monitoring tumors.

A shift to aerobic glycolysis in cancer cells is usually accompanied by a reduction in OXPHOS, achieved through unknown mechanisms. Thus, identifying oncogenic processes that directly affect mitochondrial function in general and oxidative phosphorylation in particular are critical to our understanding of these complex metabolic reprogramming events. As described in this thesis, we have begun a study aimed at understanding whether the production of the oncometabolite, 2-hydroxyglutarate, as a result of a gain-of-function mutation commonly found in different tumors, plays a role in reprogramming metabolism through modification of the mitochondrial genome.

Role of Mitochondria in disease

Dr. Warburg also postulated that the mitochondria found in cancerous cells are abnormal and become dysfunctional leading to alterations in the energy producing pathway of these cells.

While much study has been put into understand the energy portion of the Warburg effect, understanding of the mitochondrial aspect of his theory has remained somewhat lacking over the past few decades and continues to be controversial as some reports claim that mitochondrial function remains normal after tumorigenesis and OXPHOS is uninterrupted (Ward & Thompson, 2012).

The endosymbiotic theory states that the mitochondrial genome developed from a bacterial species that was engulfed by a primitive eukaryotic cell and which has retained a key role in ATP production in eukaryotes to this day. While most of the genetic material encoded by this ancestral endosymbiont has been transferred to the eukaryotic nuclear genome, several key enzymes remain encoded on the mitochondrial genome, along with rRNAs and tRNAs required for mitochondrial transcription and translation, which more closely resembles that of prokaryotes. The human mitochondrial genome, as mentioned earlier, is 16.6 kilobases in length and encodes 13 proteins integral to the function of the electron transport chain and therefore generation of ATP in the cell. Of these thirteen proteins, 7 are included in Complex I, 1 is included in Complex III, 3 are part of Complex IV and 2 are found in Complex V. One reason why these 13 proteins are retained on the mitochondrial genome is that they are highly hydrophobic and are difficult to import into the mitochondria from the nucleus. Another proposal of this theory is that these proteins are vital to the metabolic control of the cell and are thus regulated by the redox status of the cell (3) which is further emphasized by the relationship that exists between reactive oxygen species production and mitochondrial dysfunction.

The remaining proteins required for oxidative phosphorylation are encoded on the nuclear genome, pointing to a need for coordinate control of gene expression between mitochondria and nucleus. Epigenetic mechanisms are critical for regulation of gene expression

in the nucleus. Mitochondrial epigenetic mechanisms, recently described by our laboratory (68) have been implicated in altering mitochondrial gene transcription, and others have suggested a role for epigenetics in a retrograde signaling capacity between mitochondria and nucleus (69). These proposed interconnections led us to hypothesize that cancer related mutations in genes involved in intermediary metabolism, might play a role in dysregulating the balance between nuclear and mitochondrial gene expression (48).

During oxidative phosphorylation, electron carriers found in Complex I and Complex III are able to pass an electron to an O_2 molecule creating the superoxide free radical $^{\cdot}O_2^-$. This free radical is highly reactive and can produce the hydroxyl free radical $^{\cdot}OH$ as well which can damage metabolic enzymes, membrane lipids and nucleic acids. To keep free radical concentrations low, cells utilize the superoxide dismutase enzyme to convert these reactive oxygen species to oxygen and hydrogen peroxide which is then converted to water and rendered harmless by the glutathione peroxidase enzyme. Unfortunately, in some instances, ROS production is elevated beyond the level that these enzymes can control and can have serious pathologic effects on mitochondria function. Also of importance is that the mitochondrial DNA polymerase (mtPOLG) lacks a superb proofreading ability so that mutations in the mitochondrial genome arise over time (75). These two problems can lead to mitochondrial dysfunction, and can cause muscle weakness, decreased psychomotor skills and decreased bone density, signs typical of the ageing process. This buildup of ROS species and mitochondrial mutations has been implicated in various neurodegenerative diseases as well in Alzheimer's disease, Parkinson's disease, and Huntington's disease.

Transcription of the Mitochondrial Genome

While the 13 protein-coding genes are integral to the oxidative phosphorylation pathway, the mitochondrial genome also encodes 2 ribosomal RNAs and 22 transfer RNAs. Early *in vitro* studies performed in human cells using 5' and 3' end labeling of mitochondrial transcripts showed that two separate polycistronic messages are encoded on the mitochondrial heavy strand while one message is encoded on the light strand of the genome (53). One message encodes the two ribosmomal RNA genes, 12S and 16S, from a promoter termed HSP1 (Heavy Strand Promoter), located 16 base pairs upstream of the gene that encodes the phenylalanine-specific tRNA. In fact, the rRNAs are transcribed 10 to 30 more often than the protein-coding genes in the mitochondrial genome. These ribosomal components assist in the translation of the protein-coding genes which are transcribed from the second start site, HSP2. This promoter initiates transcription near the 5' end of the 12S rRNA gene and encodes essentially the entire heavy strand. A third message is initiated on the light strand from promoter LSP (Light Strand Promoter) that leads to the generation of short transcripts that act as primers in DNA replication, as well as a polycistronic mRNA that encodes the ND6 protein, a component of Complex I, and multiple tRNAs (20). Processing of these transcribed messages is proposed to take place via the tRNA punctuation model. This model suggests that tRNA secondary structures, within polycistronic mRNAs, act as molecular punctuation marks to signal for endonucleolytic cleavage before and after the tRNA messages where the protein-coding genes reside (57).

Mitochondrial gene transcription is regulated by genes encoded in the nucleus which give rise to the mitochondrial RNA polymerase (POLRMT), mitochondrial transcription factors B1, B2 and A (TFB1M, TFB2M and TFAM respectively) and mitochondrial termination factor (mTERF). These factors work together to initiate transcription in a site-specific and species-

specific manner. Much work remains to determine how these proteins interact exactly with the mitochondrial genome. One study aimed at uncovering the role of mTERF, found that this protein can initiate and terminate transcription of the rRNA transcript by binding the DNA at both the HSP1 promoter and at the end of the 16S rRNA gene causing a looping out effect of the rRNA genes, and allowing for the recycling of the transcription apparatus.

A single mitochondrion in a human cell contains, on average, five mitochondrial genomes while each cell can contain anywhere from 100 to 1000 mitochondria (64), with cultured human cells containing an estimated 8000 mtDNA genomes (13). These mitochondrial genomes allow for a more fine-tuned response to environmental stimuli outside and within the cell and make it possible to respond very rapidly to stimuli such as growth and division where energy requirements are high. Packaging of multiple genomes in the mitochondria is achieved by the TFAM transcription factor, as well as mtSSB and Twinkle helicase (22), proteins necessary for mitochondrial DNA replication which occurs independently of nuclear DNA replication. This packaging produces small structures called nucleoids which are tethered to the mitochondrial inner membrane and act in much the same way as histones do for nuclear DNA as TFAM is integral to mitochondrial DNA transcription and replication (19).

The research presented in this thesis was carried out to determine if the production of the oncometabolite, 2-HG, had an effect on mitochondrial function via an altered epigenetic pattern. Effective transcription of the mitochondrial genome through proper epigenetic modifications could possibly be necessary to carry out metabolic processes in the cell and transcription of the mitochondrial genome will be studied in future experiments to determine if this is the case. In this thesis, we have proposed a novel function of 2-HG in affecting the 5-hydroxymethylcytosine levels of the mitochondria which may lead to altered mitochondrial gene transcription impairing

cellular metabolism. This work will assist in determining the function of 5-hydroxymethylcytosine in the mitochondria and determine how the level of this modification affects mitochondrial gene transcription and metabolic function.

Discovery of a novel oncogene: Isocitrate Dehydrogenase

In an effort to determine genetic alterations in glioblastoma, one of the most aggressive and most common brain cancers in humans, DNA sequencing has been employed to determine which genes of the genome become mutated and act as tumor suppressors or oncogenes. At the time, one gene of interest that became mutated recurred over and over again in multiple tumor samples, the gene that encodes the Isocitrate Dehydrogenase 1 enzyme, a key enzyme of the TCA cycle. The most commonly found mutation of IDH1 was found to affect an arginine residue in the active site of the enzyme (59). In fact, almost all of the grade II/III gliomas as well as many secondary glioblastomas contained a mutation in the active site of the IDH1 enzyme, the enzyme that locates to the cytoplasm and peroxisomes of the cell. This mutation of IDH1, which converts the molecule isocitrate into α-ketoglutarate, a metabolite necessary for many cellular processes, was found to be heterozygous, with retention of a wild type allele, and therefore proposed to be autosomal dominant. This mutation was originally thought to result in a buildup of precursor molecules in the cell much the same way as the loss of function mutations found in the Fumarate Hydratase (FH) and Succinate Dehydrogenase (SDH) enzymes. FH and SDH occur in a single allele in the germline and tumors show a loss of heterozygosity with loss of expression of the remaining allele, producing tumors functionally null in these enzymes (89). When mutated, these enzymes lead to a buildup of precursor molecules, fumarate and succinate, which stabilize the HIF-1α protein and promote angiogenesis. These enzymes are also proposed to be inhibitory to α-ketoglutarate enzymes as well. IDH2, the mitochondrial isoform of this

enzyme, was also found to be mutated in many brain cancers as well in acute myeloid leukemia. Of particular interest is the fact that IDH1 and IDH2 mutations have been shown to be mutually exclusive of each other and do not occur simultaneously in the same sample leading many to propose these mutations have a similar function (21). They are also mutually exclusive to TET2 mutations. When analyzed, these IDH mutations were found to be heterozygous and retain a wild-type allele while the other is mutated. To date, no IDH mutations have been reported to be homozygous. This heterozygous nature has prompted many to speculate that these IDH enzymes may act as oncogenes and that these mutations result in a novel gain of function. This speculation was supported when levels of a metabolite, 2-hydroxyglutarate, were found to be dramatically increased in glioblastoma cells *in vitro* when mutant IDH1 was expressed (15).

In normal human cells, the concentration of 2-HG in the cell is maintained at an extremely low level around 0.1nM. This molecule is a by-product of a transhydrogenase reaction and is usually converted to α-ketoglutarate fairly quickly by 2-hydroxyglutarate dehydrogenase, a nuclear encoded protein. When these 2-HGDH enzymes become mutated, 2-HG builds up in the cell and results in a disease termed 2-hydroxyglutaric aciduria which can lead to mental retardation, leukoencephalopathy, and a decline in psychomotor skills. In IDH mutant patients, the levels of 2-HG in serum can rise from 0.1nM to between 1 and 30mM (17) and the (D)-isoform of this metabolite is solely produced which is typical of most enzymatic reactions. Experiments using the (L)-isoform of this metabolite have shown that it is actually a stronger inhibitor of α-ketoglutarate dependent enzymes than the (D)-isoform and is more potent in disrupting histone demethylases' as well as TET enzymes' normal functions (86).

When 2-hydroxyglutarate is allowed to build up in the cell, the oncometabolite competitively inhibits the enzymes that utilize α-ketoglutarate to function much in the same way

that the buildup of fumarate and succinate can lead to decreased enzymatic activity of these enzymes. Production of 2-HG by mutant metabolic enzymes such as IDH, can also lead to reduced levels of α-ketoglutarate as this molecule is a common analog of the necessary metabolite. To date, more than 70 different enzymes utilize α-ketoglutarate as a substrate. These enzymes include prolyl hydroxylases, histone modifying enzymes, as well as DNA methylcytosine dioxygenases and other α-ketoglutarate dependent enzymes which can have a profound effect on gene expression. The activity of prolyl hydroxylases was inhibited following the introduction of IDH2 mutations in cell culture models leading to greater stability of their target molecule, HIF-1α. Consequently, when IDH2 is mutated, stem/progenitor cell marks (C-kit) increase while markers indicative of mature hematopoietic cells (Gr-1 and Mac-1) became reduced in the cell indicating a profound effect on cellular differentiation by the competitive inhibition of these enzymes by 2-hydroxyglutarate (21). Mutant IDH enzymes also impair histone demethylation in a 2-HG dependent manner and have increased levels of repressive histone modifications such as H3K9me3 leading to a block in cellular differentiation (49). Inhibition of DNA-modifying enzymes also occurs when 2-hydroxyglutarate is elevated in the cell. Of these enzymes, the Ten-eleven Translocation (TET) proteins are integral members which catalyze the conversion of 5-methylcytosine residues in the nuclear genome to 5-hydroxymethylcytosine (72). This novel cytosine modification has been thought to be integral to the DNA demethylation pathway as it can further oxidize modified cytosine residues to 5-formylcytosine and 5-carboxylcytosine. Utilizing AID deamination and the base excision repair pathway, this carboxyl modification can be converted to an unmodified cytosine base.

The TET family of enzymes, of which there are three members, utilize Fe^{2+}, O_2 and α-ketoglutarate to catalyze the conversion of 5mC to 5hmC and can subsequently continue to

oxidize the 5hmC modified base to 5-formylcytosine and finally 5-carboxylcytosine which can then be deaminated to thymine and removed via the base excision repair machinery of the cell (32, 26). This active DNA demethylation pathway has been proposed as a way of removing the methylation marks on cytosine residues of transcriptionally repressed promoters and may therefore lead to the re-expression of silenced genes. As the TET enzymes require α-ketoglutarate, it has been proven that a surplus of 2-hydroxyglutarate can competitively inhibit these enzymes, as well as possibly reduce the pool of α-ketoglutarate available within the cell, leading to a decline in the amount of 5-hydroxymethylcytosine residues present in the nuclear genome and a consequent rise in the abundance of genomic 5-methylcytosine. This results in the establishment of the CpG island hypermethylator phenotype (CIMP) in these IDH mutant samples (21, 76).

Other effects of (D)-2-hydroxyglutarate production by mutated IDH proteins have been determined to affect other aspects of the cell. When IDH1 is mutated, studies have shown that collagen maturation is impaired due to inhibition of collagen proline-hydroxylases which also may act as tumor suppressors in B-cell lymphomas. DNA repair enzymes also seem to be affected by 2-HG as well as RNA demethylases that regulate cellular metabolism.

The predicted role of mutant IDH in human mitochondria

Recently, the Taylor lab identified the presence of the DNMT1 maintenance methyltransferase in the mitochondria, translocated using a mitochondrial targeting sequence (MTS) encoded from an upstream start codon. The presence of a MTS was conserved among mammalian species, and was shown to translocate a heterologous protein (GFP) into the mitochondria. This finding helped to solidify the fact that cytosine modifications can exist in the

mitochondria as well as in the nucleus in the context of CpG sites in the mitochondrial genome by a mitochondrial isoform of DNMT, mtDNMT1. Interestingly, this mtDNMT1 isoform is up-regulated by PGC1α and NRF1, necessary molecules for the oxidative stress response and for mitochondrial biogenesis (65, 84), and down-regulated by p53, a tumor suppressor gene mutated in over 50% of all human tumors. Loss of p53 function in cells leads to a modest 3-fold increase in total DNMT1 transcript but a 6-fold increase in the abundance of mtDNMT1 gene transcript. Through the use of the MeDIP technique as well as the β-glucosyltransferase assay used to detect 5-hydroxymethylcytosine in DNA, it was determined that 5-hydroxymethylcytosine, as well as 5-methylcytosine residues are present in the mitochondrial genome (68). Furthermore, increased expression of mtDNMT1 resulted in a gene-and-strand specific alteration in mitochondrial transcription. The conservation of the enzymes and modifications resulting from their action within mitochondria has led to the hypothesis that mitochondrial gene transcription might be regulated through epigenetic mechanisms.

With this information, the research described in this thesis was undertaken to determine whether mutant IDH1, IDH2 or both isoforms have any effect on the patterns of methylation and hydroxymethylation found in the human mitochondrial genome. As is shown in Chapter 3, when either the IDH1 or IDH2 mutant enzymes are transfected into human cell lines, a phenomenon similar to that seen in the nucleus is found in regards to hydroxymethylation most likely through the inhibition of TET enzymes by 2-hydroxyglutarate. After 48 hours, we show that expression of mutant IDH1 or IDH2 causes a substantial decline in the level of 5-hydroxymethylcytosine to about 40-50% that of the wild-type IDH transfected samples. For the experiments performed in this thesis, both the 12S and 16S rRNA gene regions were first analyzed to determine if mutant IDH affects levels of modified cytosine residues in the mitochondria. This effect is most notable

within the 12S ribosomal RNA region of the mitochondrial genome, but less apparent in the 16S rRNA region of the genome where levels of 5hmC may remain unchanged or even increase in the presence of IDH mutations. Future studies investigating the other mitochondrial genes will be performed to see if a similar trend is produced across the entire mitochondrial genome after mutant IDH is expressed. It is possible to infer that when IDH1 or IDH2 are mutated in human cell cultures, the levels of 2-hydroxyglutarate produced by these mutations rise above their normal cellular levels and competitively inhibit the TET enzymes as the experiments performed in this thesis were done exactly the same as those reported earlier (86, 21). This inhibition in the active site of the enzyme results in fewer 5-hydroxymethylcytosine residues converted from existing 5-methylcytosine residues in the mitochondrial genome. Impressively, these levels of 5hmC are reduced fairly quickly, after only 48 hours post-transfection, with mutant IDH, showing that the effects of IDH mutants can be felt in the mitochondrial genome extremely rapidly and suggesting that the stores of α-ketoglutarate are under tight control within the cell. Also of interest is that when these mutant IDH isoforms are co-transfected with the D-isoform of 2-hydroxyglutarate dehydrogenase (2-HGDH), the levels of 5hmC begin to rise back to the levels seen in IDH wild-type transfected samples showing that this enzyme can relieve the inhibition on the TET enzymes by the 2-HG oncometabolite, presumably through conversion of 2-HG to α-KG. While rescue is clearly observed in the IDH1 transfected samples, the levels of 5hmC found in the IDH2 mutant transfected samples tend to decrease when co-transfected with the D-2HGDH enzyme. To accurately determine how this enzyme affects 2-HG levels, mass spectrometry should be employed to measure the level of this oncometabolite after co-transfection of (D)-2HGDH. As α-ketoglutarate is not as limited in the mitochondria as it is in the nucleus, the level of 2-HG produced by this mutation could be substantially larger than that

produced by the IDH1 mutant enzyme and should be measured using mass spectrometry to accurately determine the levels of this metabolite in the cell. In fact, reports have demonstrated that the concentration of 2-HG is much higher in IDH2 mutated samples than IDH1 mutant samples which may possibly be saturating the 2-HGDH enzyme which can travel between the mitochondria and cytoplasm.

While the effects of IDH mutation on 5hmC content in the mitochondrial genome can be determined fairly easily, how IDH1 and IDH2 mutant enzymes affect the levels of 5-methylcytosine in the rRNA regions of the genome is less clear. As a result of the decrease in 5hmC seen in these transiently transfected cells, we expected to see a concomitant increase in 5mC. As can be seen from the results in Chapter 3, when IDH1 mutant enzymes are introduced into HEK293 cells, the level of 5-methylcytosine in these samples increases in both the 12S and 16S regions of the genome. However, in the IDH1 and IDH2 wild-type samples, the levels of 5mC are well below the 5mC levels of their respective IgG mouse control levels which is used as a measure of baseline. When analyzing the IDH2 R172K mutation, the levels of 5mC, as a measure of percentage of input, in both the 12S and 16S region decrease below the IgG mouse control which is opposite to the effect this mutation has on the nuclear genome. There are several technical factors that could contribute to the inconclusive nature of these results. The performance of the mouse IgG control antibody shows a very high degree of batch-to-batch variability. We have tested two different lots of mouse IgG. The first lot consistently gave backgrounds that were substantially above 5mC antibody signal. The control IgG used in the experiments described in this thesis was somewhat better in performance, but still gave higher background than those previously reported (68). Others have reported that alterations in nuclear 5mC, as a result of transfection with IDH mutants, occur slowly over many cell generations (76).

We have accordingly isolated clones of stably transfected cells expressing varying levels of mutant IDH proteins to determine whether a similar phenomenon occurs in the mitochondrial genome. Co-transfection of the IDH1 mutation with the D-2HGDH enzyme leads to a decrease in the levels of 5mC content when analyzing the 12S and 16S rRNA regions, while co-transfection of the IDH2 mutant enzyme leads to an increase in 5mC content. With this confounding evidence, it is difficult to determine how IDH mutations affect the 5-methylcytosine levels of the mitochondrial genome. Recently, a positive correlation has been determined between mutant IDH1 and p53 expression as lower grade gliomas, which typically harbor the IDH1 R132H mutation, also show an increase in p53 levels which is a commonly mutated protein found in cancers (7, 73). This interaction seems to provide evidence that mitochondria are integral to cell cycle progression (40) although the functional significance of this interaction remains to be determined. With the evidence produced by the Taylor lab regarding the effects of p53 on mtDNMT1 transcription, it is probable that a connection exists between mutant IDH and mtDNMT1 expression. Future studies will determine how mutant IDH affects the expression level of mtDNMT1 to find if a connection truly exists between this oncogene and the methylation machinery of the mitochodnria.

To better understand how and if the mitochondrial rRNA regions become hypermethylated, stable clones were produced that express either the wild-type or mutant enzymes of the IDH isoforms. In the nuclear genome, it was determined that hypermethylation of both cytosine residues and histone markers does occur slowly over many cell generations when mutant IDH is introduced (about 20 passages of HEK293 cells) (49). How these IDH mutations may come to affect the mitochondrial genome over time remains to be determined and we are currently working to solve this question. Stable clones have been produced and analyzed using

immunoblotting techniques. As was shown in Chapter 3, the clones chosen express their transfected mutant IDH protein at a level equivalent to or similar to the endogenous levels of IDH in HEK293 cells when using an antibody specific for IDH proteins. We have also chosen clones that overexpress mutant IDH to varying degrees. Future MeDIPs and hydroxy-MeDIPs will be performed at different passages of the cells to determine if the levels of 5-methylcytosine rise substantially above baseline in the mitochondrial genome or whether a different effect is seen in regards to mitochondrial cytosine modifications when IDH mutant proteins are expressed stably within the cell population.

The Importance of Mitochondrial Cytosine Modifications

It has been shown that not only is TET2 present in the mitochondria but also the enzymatic machinery necessary for the base excision repair pathway indicating a role for 5-hydroxymethylcytosine in DNA demethylation and gene re-expression in the mitochondria that is similar to that found in the nucleus. Also, as mitochondria contain protein-containing nucleoid structures that package the DNA with the high-mobility group protein TFAM and other proteins, methylation and hydroxymethylation may be integral in the stability of these higher order structures and therefore help to control transcription of mitochondrial genes. What's more, this control could be specific to certain mitochondrial genes as changes in gene transcription occur differentially in different areas of the genome as a result of changes in mtDNMT1 levels (68). These results point toward mitochondrial cytosine modifications as a possible biomarker and diagnostic tool for disease (30).

Studies have determined that Complexes IV and V can also be inhibited by the oncometabolite, 2-HG, and lead to superoxide production and decreased cell viability (43). It is

possible that this inhibition is caused by methylation of the genes encoding proteins necessary for these complexes although further work remains to be done. The effect IDH1 and IDH2 mutations have on the 12S rRNA region of the mitochondrial genome is intriguing. Methylation of this area has been implicated in disease and ageing phenotypes. One epidemiological study determined that when workers were exposed to airborne pollutants, methylation levels of the 12S rRNA region increased compared to subjects not exposed to these same pollutants (Byun, 2013). Another study measured the level of 12S methylation in adult males and found that the percentage of methylation in this region decreased with age (Giordano, 2012). While this work remains to be replicated, it is interesting that methylation of this region may play such an important role in human health and could possibly become a target of therapeutics. By comparison, work regarding how methylation of the 16S rRNA region of the mitochondrial genome, which is downstream of the 12S region, has been lacking and will require future experiments to determine if modification of this rRNA gene is significant to overall mitochondrial function.

Just as important as methylation status and cellular differentiation, mutation of IDH enzymes could affect cellular metabolism as well. When mutated, the defective allele of IDH utilizes α-ketoglutarate and NADPH to produce 2-hydroxyglutarate. This results in a net reduction of available NADPH in the cell decreasing the $NADPH/NADP^+$ ratio (Bleeker, 2010). This molecule, which functions as an electron acceptor for the oxidative phosphorylation pathway, is vital to macromolecular biosynthesis and serves as a powerful antioxidant (37). Many anabolic pathways employ NADPH as a co-factor in the synthesis of nucleic acids, protein and lipids (10). As mentioned previously, NADPH serves as a co-factor for glutathione reductase to clear the cell of harmful free radicals. Dysregulation of the cellular redox status can contribute

to cellular transformation and tumorigenesis. Although how this redox status is affected in IDH-mutant tumors remains to be determined, studies suggest IDH provides a protective role (8, 44).

Perspectives and Conclusions

The effects of IDH mutations in the cell are multifaceted. This novel oncogene serves to dysregulate both cellular gene expression and differentiation. The involvement of mutant IDH and the oncometabolite produced in cellular metabolism is of considerable interest and is the focus of work arising from this thesis. Cell culture work has determined that this enzyme, when mutated, leads to an increase in a metabolite, (D)-2-HG, which competitively inhibits enzymes that utilize α-ketoglutarate in the cell, the metabolite normally produced by IDH (86). This leads to increased gene methylation over time via inhibition of TET proteins in the nucleus (76, 21). In this thesis, we determined the effect of this oncometabolite on the mitochondrial genome, in which cytosine residues have been shown to be both methylated and hydroxymethylated. The results obtained clearly show an effect of 2-HG on hydroxymethylation of specific mitochondrial genes. Further work will need to be done to determine how this mutated enzyme affects methylation levels and how these different methylation patterns affect mitochondrial function and mitochondrial gene transcription. As described by Nass, methylation in the mitochondria is quite sparse and tightly regulated (54). Also, how NADPH reduction affects cellular metabolism will be vital to understanding this enzyme's function. To determine the functional consequences of decreasing 5hmC levels, abundance of mitochondrial transcripts will be analyzed to determine if this modification has an effect on gene expression in this subcellular compartment. Also, the new XF Analyzer technology developed by Seahorse Bioscience could be utilized to measure mitochondrial function in individual cells by determining oxygen consumption and extracellular acidification as a function of respiration and glycolysis respectively. If IDH mutations affect

gene expression through decreased hydroxymethylation in the mitochondrial genome as well as decreasing NADPH stores in this organelle, we would expect that respiration might be less effective in cells harboring this mutation. This may thus push cells towards a more glycolytic state thereby increasing the amount of lactate produced by the cell since ATP will not be produced as rapidly as needed.

The mitochondrial genome accounts for less than 1% of the total DNA found in human cells. However, the information encoded on this genome is integral to the health and viability of human cells. While it remains unclear how epigenetic markers affect the mitochondrial genome, it is interesting to speculate about the role of both 5-methylcytosine and 5-hydroxymethylcytosine in both mitochondrial maintenance and transcription. Our work suggests an important interplay between epigenetics and the metabolism of the human cell. Dr. Warburg's work, published more than half a century ago, has taken on a new importance as our understanding of the relationship between misregulated metabolism and carcinogenesis expands. With the work performed in this thesis, we have expanded our understanding of epigenetic factors in the mitochondria and opened the way to a greater understanding of the impact of aberrant metabolic enzymes on the mitochondrion and the cell. A new area of research into mitochondrial epigenetics has been opened linking metabolism and cytosine modification together via the mutation of the isocitrate dehydrogenase gene.

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Vita

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